Universidade de Aveiro Departamento de Biologia
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José Manuel Carita Gonçalves

Photosensitizer effect in *Aeromonas salmonicida* lipids
Efeito do fotosensibilizador nos lípidos de *Aeromonas salmonicida*

DECLARAÇÃO

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Maria Adelaide de Pinho Almeida, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro e da Doutora Maria do Rosário Gonçalves Reis Marques Domingues, Professora Auxiliar com Agregação do Departamento de Química da Universidade de Aveiro

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palavras-chave

Inactivação fotodinâmica, *Aeromonas salmonicida*, oxidação, lípidos, ácidos gordos, hidroperóxidos, fotosensibilizadores, porfirinas, inactivação bacteriana

resumo

A Inactivação fotodinâmica é um método simples e eficiente na inactivação de microorganismos. Inactivação fotodinâmica combina o uso de luz com um fotosensibilizador, como por exemplo porfirinas, que na presença de oxigénio gera a formação de espécies reactivas de oxigénio, como o oxigénio singleto e radicais livres, que são capazes de oxidar componentes membranares vitais. As principais vantagens da Terapia Fotodinâmica (TFD) são a sua eficiência na inactivação de bactérias, fungos, leveduras e protozoários; o baixo nível de indução de resistência; e o uso de fontes de luz baratas. Para melhor compreender esta técnica, é fundamental compreender o seu mecanismo de acções em alvos celulares. Os lípidos são importantes componentes nas membranas bacterianas, que muito recentemente foram reconhecidos como um dos alvos da PDI, e que podem estar envolvidos no processo de inactivação bacteriana. O principal objectivo deste estudo foi avaliar o efeito de quatro derivados porfirínicos utilizados com fotosensibilizadores em PDI, na foto-oxidação de lípidos membranares em *Aeromonas salmonicida*, e relacionar este efeito com a inactivação desta bactéria. Para tal foram realizados testes para a avaliação da peroxidação lipídica, através da quantificação de hidroperóxidos lípidos por FOX II e pela análise da variação do perfil de ácidos gordos por GC-FID em diferentes tempos de exposição à luz. Os resultados obtidos foram correlacionados ensaios de viabilidade celular;

Após PDI foi observada a formação de hidroperóxidos lipídico, alterações no perfil de ácidos gordos e diminuição da sobrevivência celular. No entanto estes resultados estão dependentes na escolha da porfirina, tal como outros estudos demonstram. Foi possível estabelecer uma relação directa entre a foto-oxidação dos lípidos membranares com a foto-inactivação da bactéria em estudo e estabelecer uma ordem de eficiência para as quatro porfirinas.

Este estudo vem reforçar que as porfirinas catiónicas são eficientes na inactivação de bactérias e que a inactivação fotodinâmica é importante e eficiente, sendo uma técnica viável alternativa a metodologias tradicionais.

keywords

Photodynamic therapy, *Aeromonas salmonicida*, oxidation, lipids, fatty acids, hydroperoxides, photosensitizers, porphyrins, bacterial inactivation

abstract

Photodynamic inactivation is a simple and effective method to destroy microorganism. PDI combines the use of light with a photosensitizer, as porphyrins, which in the presence of oxygen, leads to the formation of reactive oxygen species, such as singlet oxygen and free radicals, capable to oxidize vital membrane structures. The key advantages of Photodynamic Therapy (PDI) are the efficacy in bacteria, fungi, yeasts and protozoa; the low level of resistance induction; and the use of a cheap light source. It is fundamental to understand the importance of cell targets to better understand the photo-oxidation process. Lipids are important membrane components in bacteria. The main goal of this study was to evaluate the charge effect of four porphyrin derivatives used as photosensitizers in PDI in the photo-oxidation of membrane lipids of *Aeromonas salmonicida* and relate with the inactivation of this bacterium. The goal was achieved by quantification of lipid hydroperoxides by FOX II method, fatty acid profiles analysis by GC-FID and viability assays; in different periods of light exposure.

After PDI it was observed formation of lipid hydroperoxides, changes in the fatty acids profile and a decrease on cell survival. However the results are dependent on the porphyrin used. According to these results, the photo-oxidation is not directly proportional with the number of charges in the photosensitizers, as other studies had been reported. A direct relation between the photo-oxidation of membrane lipids with the photo-inactivation in the studied bacterium was observed and an order of effectiveness was established. This study reinforces that cationic porphyrins are effective to inactivate bacteria and the importance and efficiency of photodynamic inactivation, as a viable alternative to traditional procedures.

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List of Acronyms and Abbreviations

ºC	Degree Celsius
µL	Microliter
µM	Micromolar
mL	Millilitre
A.salmonicida	*Aeromonas salmonicida*
ANOVA	Analysis of variance
Di-Py^+-Me	5,10-bis(1-methylpiridinum-4-yl)-10,20-bis(pentafluorophenyl) porphyrin diiodide
E.coli	*Escherichia coli*
Gram –	Gram negative
Gram +	Gram positive
LOOH	Lipid hydroperoxide
LPS	Lipopolysaccharide
Mono-Py^+-Me	5,10,15-tris(pentafluorophenyl)-20-(1-methylpiridinum-4-yl) porphyrin iodide
PDI	Photodynamic Inactivation
PDT	Photodynamic therapy

PS	PS
rpm	Revolutions per minute
Tetra-Py^{+}-Me	5,10,15,20-tetrakis(1-methylpiridinium-4-yl) porphyrin tetra-iodide
Tri- Py^{+}-Me	5-(pentafluorophenyl)-10,15,20-tris(1-methylpiridinium-4-yl) porphyrin triiodide
TSA	Triptic soy agar
TSB	Triptic soy broth

Chapter I

Introduction

1. Photodynamic Therapy: history, a brief review and applications

The discovery of antibiotics was a remarkable and colossal step for science. This prompted an enormous improvement in medicine, leading to a more effective treatment of microbial infections, even in diseases considered as irredeemable. Antibiotics interfere with the bacteria's ability to repair its damage DNA, stopping the bacteria's ability to make what it needs to grow new cells or by weakening bacteria's cell wall until it rushes (Godzeski et al., 1967). For decades, antibiotics were described and understood as capable to lead a major decline of microbial diseases worldwide. Due of their specific targets in pathogenic microorganisms, antibiotics are capable to effectively inactivate microorganisms (Jori et al., 2006; Tulkens, 1991). The lack of knowledge about the resistance mechanisms of microorganisms and the inadequate use of antibiotics caused an increase resistance of microorganisms for multiple drugs (Winckler, 2007). The inappropriate prescription of antibiotics with broad spectrum and the failure of some patients to complete their treatment also exacerbate the problem (Cassell GH and Mekalanos J, 2001). The research for sustainable alternatives for antibiotics is currently a priority (Almeida et al., 2009; Sievert et al., 2008), not only in clinical field, but also in environmental areas and food industry (Riley, 1994). Photodynamic Therapy (PDT) appears in the past years as a viable alternative, cost efficient and with promising applications in several areas, namely, as an antimicrobial approach, in clinical field, food industry and environmental control (Sievert et al., 2008; Costa et al., 2011; Alves et al., 2009a)

Although the main use of PDT was in the treatment of localized cancers, the worldwide emergence of antibiotic resistance amongst pathogenic bacteria has led to a major research effort to find alternative antibacterial therapeutics. Drug resistance in bacteria might be inherited or acquired through process of gene transfer or genetic mutation. Some bacteria are inherently resistant to some classes of antibiotics or to one in particular, as an example, all Gram negative bacteria are resistant to glycopeptides and Gram positives are resistant to aztrenonam. This resistance may occur at the level of permeability to the

particular antibiotic or at the target site (Barker, 1999). There is, however, a large variety of mechanisms by which bacteria can enhance to external threats, as thickening out outer wall, encoding new proteins preventing drug penetration, advent of mutants deficient on porin channels that would allow the influx of externally added chemicals (Jori, 2006).

Important bacteria resistance structures in Gram-negative bacteria are efflux systems, which are able to derivate various antimicrobial agents such as antibiotics, biocides, dyes and detergents (Poole, 2001). Intrinsic or acquires resistance depends on restrained drug gathering and/ or antimicrobial modification or destruction (Hancock and Bell, 1988).

The knowledge behind PDT results from a vast and intense work that was made over the years, increasing the information, the efficacy and the applications of this therapy. From very early on, the light has been studied and used for therapeutic purposes. Civilizations, such as Greece, Egypt and India, have used phototherapy in the treatment of psoriasis, vitiligo, rickets and skin cancer (Ackroyd et al., 2001; Spikes, 1989). Regardless it very old application, this procedure and knowledge was not in use until the early twentieth century. Only in 1900, the researcher Oscar Raab rediscovered that this therapy could be able to induce cell death trough the interaction between light and a chemical compound, nowadays designated as photosensitizer (PS) and oxygen (Daniell and Hill, 1991; Ackroyd et al., 2001). Raab discovery led to the development of, what is currently known in general as Photodynamic Therapy, when used for cancer and other diseases, or Photodynamic Inactivation (PDI), when used to inactivate or kill microorganisms (Tavares et al., 2010; Ackroyd et al., 2001).

The full potential of PDI, as an antimicrobial agent, was not explored for several decades (Ackroyd et al., 2001), mainly because of the antibiotics discovery and usage, that had proved to be extremely efficient in microbial infections treatment and it was thought, even if incorrectly, that the use of these compounds would gradually lead to a significant reduction of microbial diseases (Daniell and Hill, 1991). Another aspect that delayed the study and application of PDT was the ineffectiveness against bacteria with more complex walls, particularly in gram negative bacteria. These unsuccessful results appeared due to the use of neutral or negative charged PSs alone, which are not the most efficient compounds according to more recent studies (Costa et al., 2011; Alves et al.,

2009a).

Summarizing, PDT combines the use of light with a PS, which in the presence of oxygen, leads to the formation of reactive oxygen species (ROS), such as singlet oxygen and free radicals, such as hydrogen peroxides (Figure 1) (Patrice, 2003). Those ROS have cytotoxic properties (Riley, 1994) that are capable to oxidize many vital biological molecules, including proteins, lipids and nucleic acids (Tavares et al., 2010).

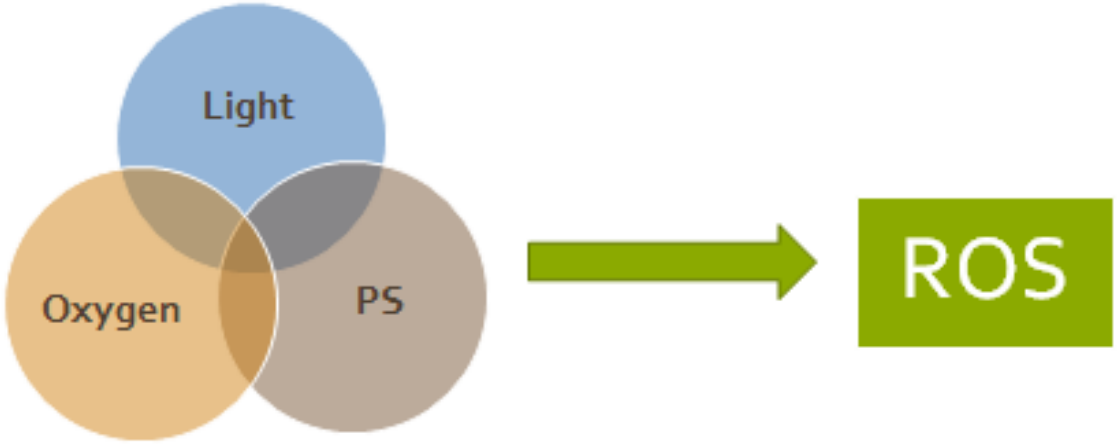

Figure 1 – Schematic representation of light, oxygen and a PS interacting to create reactive oxygen species, such as singlet oxygen and free radicals.

PS transfers energy from light to molecular oxygen, to generate reactive oxygen species (ROS). This reaction of energy transfer take place near the local where the PS is, resulting in localized and specific damage to the target cells (Dolmans et al., 2003). Toxic and mutagenic injuries produced by singlet oxygen can accumulate itself on the cell DNA originating mutations, degenerative diseases and cancer (Agnez-Lima et al., 2012b). Up ahead will be better explained the concept of PS, how it is used, and the importance of these compounds in PDI. PSs can be administrated by several ways; the most used in humans are by intravenous injection or topical application to the skin, because it allows an easier and fastest bond of the PS molecule to the cell target (Dolmans et al., 2003).

The oxidation process after PDI is not seemingly reversible and resistance mechanism has not so far been detected (Tavares et al., 2010; Costa et al., 2011). PS acts as a catalyser. If light and oxygen are present, many singlet oxygen and free radicals are produced by only one single molecule of PS (Jori and Brown, 2004a).

According to the literature, it is general assumed that the use of cationic porphyrins, with two or more charges, as PSs, is more effective in PDI for both Gram-positive and Gram-negative bacteria (Jori and Brown, 2004b; Arrojado et al., 2011; Alves et al., 2009a). Some PSs (as cationic porphyrins) have a phenyl ring and different substituents on this ring confer an amphiphilic character, but it does not affect significantly their photo physical properties (Reddi et al., 2002). The basic principles and mechanism of photo therapy that are in the genesis of this technology are:

a) The Grotthus-Draper law – "The light used must be of an appropriate wavelength, because only absorbed light can trigger a photochemical reaction."

b) The Stark-Einstein law – "Each molecule involved in a light-induced reaction absorbs one quantum of the light emitted."

c) Bunsen-Roscoe law – "The photochemical effect is a function of the product of the intensity of the light and the duration of the treatment." (Meisel and Kocher, 2005) (Ryder, 2002)

The key advantages of PDI are the great efficacy in different microorganisms, such as bacteria, fungi, yeasts and protozoa and the low level of resistance induction. (Jori et al., 2006; Tavares et al., 2010; Pereira et al., 2014). According to Jori et al., 2006, the main positive features of PDT and/or PDI are: a broad spectrum of action, as one PS can act on bacteria, fungi, viruses, and parasitic protozoa, being able to bond to specific targets in each of these microorganisms; efficacy independent of the antibiotic resistance pattern of the given microbial strain; possibility to develop PDT protocols, which lead to an extensive reduction in pathogen population with very limited damage to the host

tissue; lack selection of photoresistant strains after multiple treatments; small probability to promote the onset of mutagenicity; availability of formulations allowing a ready and specific delivery of the PS to the infected area; and use cost effective light sources for activation of the photosensitizing compound (Jori et al., 2006).

Many studies have been reporting that photodynamic therapy is an efficient treatment to inactivate microorganisms (Dai et al., 2009; O'Riordan et al., 2005; Sharma et al., 2011; Pereira et al., 2014; Arrojado et al., 2011), for the disinfection and sterilization of contaminated blood (Dai et al., 2009; Mohr et al., 1997) and for treatment of waste waters (Alves et al., 2008; Bonnett, 1995; (Bonnett et al., 2006; Carvalho et al., 2007. Over the past few years, environmental and human pollution has increased. This fact associated with the lack of safe and efficient techniques for wastewaters treatment has led to a decreased of available water resources (Alves et al., 2008; Carvalho et al., 2007; Bonnett et al., 2006). Currently, there is a need treat wastewaters using cheaper, efficient and ecological methodologies. A study from Alves et al. reports that photodynamic inactivation can be applied in the treatment of wastewaters under solar irradiation (Alves et al., 2008).

In a study conducted by Mohr in 1997, photodynamic therapy was used to treat plasma units by combining illumination at a visible wavelength with methylene blue as PS at a concentration of 1mM. This study reports effectiveness of this technique in the inactivation of hepatitis B, hepatitis C, human immunodeficiency virus (HIV) and porcine parvovirus B19 (Mohr et al., 1997)

1.1 Photosensitizers and their importance in Photodynamic Inactivation

A sensitizer is a compound capable of light emission, after receiving energy, which became excited previously in a chemical reaction. When the energy is provided as light, it is called Photosensitizer (PS). PS refers to any chemiluminescent compound, capable of light emission after receiving energy (Schiavello, 1985).

These molecules can be natural or synthetic compounds which undertake excitation after interaction with an appropriate light radiation. Different PSs have different wavelength range of maximum efficiency, as it is demonstrated in Table I (Wainwright, 1998). A photosensitizing agent with potentially optimal properties should be endowed with specific features, in addition to the expected photo-physical characteristics, such as a high quantum yield for the generation of both long-lived triplet state and the cytotoxic singlet oxygen species, a good absorption capacity at a wavelength of the spectrum where the light source is emitted; and a good efficacy to generate reactive oxygen species (ROS) (Rice et al., 2000) (Wainwright, 2003; Maisch et al., 2004).

Table I – Wavelength absorption range for some of the most studied compounds used as PSs (Adapted from (Wainwright, 1998)

Type of PS molecule	***Wavelength range in buffer (nm)***
Psoralen	300 – 380
Acridine	400 – 500
Porphyrin	400 - 450
Phenazine	500 – 550
Cyanine	500 – 600
Perylenequionoid	600 – 650
Phenothiazinium	620 – 660
Phthalocyanine	660 - 700

The PSs used in PDI must be highly selective and specific; consequently they can be used in low concentrations. At lower concentrations, PSs can induce damage in bacterial cells without being harmful to general eukaryotic cells, as mammalian cells for example. PSs usually are low energetic toxic compounds

(Arrojado et al., 2011; Pereira et al., 2014; Almeida et al., 2009; Almeida et al., 2011).

PSs are inactive in its fundamental state and have the ability to absorb light in the visible region of the electromagnetic spectrum. Typically, these compounds do not persist in the environment for long periods of time (Meisel and Kocher, 2005; Huang et al., 2010).

The most important and used PS belongs to the heterocyclic compounds family, such as, Phenothyazines (toluidine blue, methylene blue, among others); Tetrapyrrolic macrocycles (porphyrins, Chlorins, phthalocyanines, nafthalocyanines, among others); Psoralens (Furanocoumarias, among others); Acridines; Cyanines; Merocyanines (Patrice, 2003; Kadish et al., 2000). The majority of used PS used are derived from tetrapyrrole aromatic nucleous, found in many natural pigments (Castano et al., 2004; Almeida et al., 2011).

The PSs must have hydrophilic and hydrophobic characteristics, because, they must be administrated in a solution, but on the other hand they must be able to cross the bacterial cell wall. In the cell membranes transport system, the lipid bi-layer membrane allows the passive transport of hydrophobic molecules, i.e., small molecules that repel the water can spread in the cell membrane without the need for an active system of transport, as for example the adenosine 5' – triphosphate. Thus, the PS can spread more easily through the cell membrane, improving the efficiency of the photo-oxidation effect (Wainwright, 1998).

Other reported characteristics for an ideal PS are: they should have low levels of toxicity in the dark and low incidence toxicity; they should absorb light in a specific wavelength range (Detty et al., 2004).

The selectivity of the PS can be obtained by appropriate chemical design of the PS, which ensures that it will bind preferentially to microbial cells instead of mammalian cells. Another important characteristic during the design of a PS for PDI is their water solubility and positive charge (Dai et al., 2010), being extremely important in gram-negative bacteria since their membrane structure excludes many anionic and uncharged lipophilic molecules that would lead to phototoxicity (M. R. Hamblin and Hasan, 2004).

Although there is some good PS, just few of them are available commercially. The first PS approved for PDI was a solution with water and porphyrins. Later, a purified version of this solution, called Photofrin®, was approved. Photofrin® is

still used for PDI; it has a long phototoxicity (six to ten weeks) and low absorbance (630nm), which are significant disadvantages. Many efforts were made to produce a second generation of PSs. Benzoporphyrins (BpD-MA), meso-tetrahydroxiphelanilchlorine derivatives (m-THPC) and phthalocyanines are part of the new generation of PS (Fig. 2) (Josefsen and Boyle, 2008).

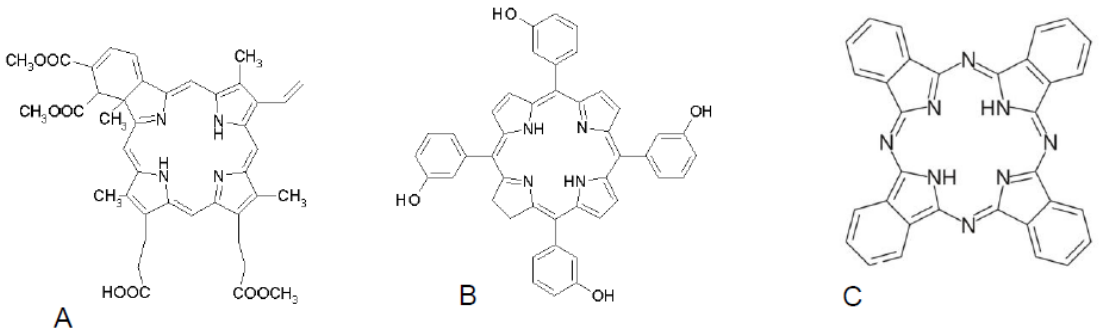

Figure 2 – Chemical representation of the new PSs – A – BPD-MA; B – m-THPC; and C – Phthalocyanine (Adapted from Josefsen and Boyle, 2008)

In short, PSs are natural or synthetic molecules that must have good absorption capacity after irradiation. They include organic dyes, such as rose Bengal, acridine orange, methylene blue; porphyrins, phtahlocyanines and related tetrapyrrolic macrocycles (M. Merchat et al., 1996; Wainwright, 1998; Rice et al., 2000; Jemli et al., 2002) The application of PDT s remarkably dependent on the PS selection. Porphyrins and their derivatives have been reported as one of the most promising compounds used in photodynamic therapy (Almeida et al., 2009; Alves et al., 2009a).

1.1.1 Porphyrins as PSs

Among the most used PSs in photodynamic therapy we can found the porphyrins (Figure 3). Porphyrins are essential in biochemical processes, such as in oxygen transport (haem) and photosynthesis (chlorophylls) (Almeida et al., 2011). These compounds belong to the class of aromatic heterocyclic compounds that are widely abundant in nature (Almeida et al., 2011; Hamblin et al., 2011). Molecules, such as haemoglobin, myoglobin, cytochromes,

chlorophylls and vitamin B, are part of the porphyrinic compounds (Stadtländer, 2013). Porphyrins consist of four pyrrole subunits linked together by four methane bridges creating a tetrapyrrole ring structure, named porphin. The derivatives of porphins are named porphyrins. Tetrapyrroles are naturally occurring pigments, which are used in many biological processes. These compounds are synthetized with uroporphyrinogen III as a common intermediate and modified to permit coordination of different metals at the ring centre, as an example, iron in heme and siroheme; or cobalt in vitamin B12 (Berg et al., 2007). Porphyrins can be synthetized into cationic units through the insertion of positively charged substituents in the peripheral positions (*meso* positions) of the tetrapyrrole macrocycle (Figure 3), which might fundamentally affect the kinetics and level of binding with bacterial cells (Jori, 2006) (Jori et al., 2006).

In the majority of the cases, porphyrins are associated with metallic ions, originating metaloporphyrins. The four nitrogen atoms located in the inner part of the porphyric macrocycle can complex with metal cations. Examples of these structures are the heme group of haemoglobin, myoglobin, cytochromes and peroxidase (important compounds for biological cells against oxygen toxicity). As mentioned previously, the first PS approved for cancer treatment by FDA (Food and Drug Administration) was the Photofrin®, a porphyrinic compound used in the treatment of lung cancer in Canada, Nederlands, Germany, Japan and United States of America (Demidova and Hamblin, 2005). The PS that was meticulous studied for PDI applications was a hematoporphyrin derivate (Boyle and Dolphin, 1996).

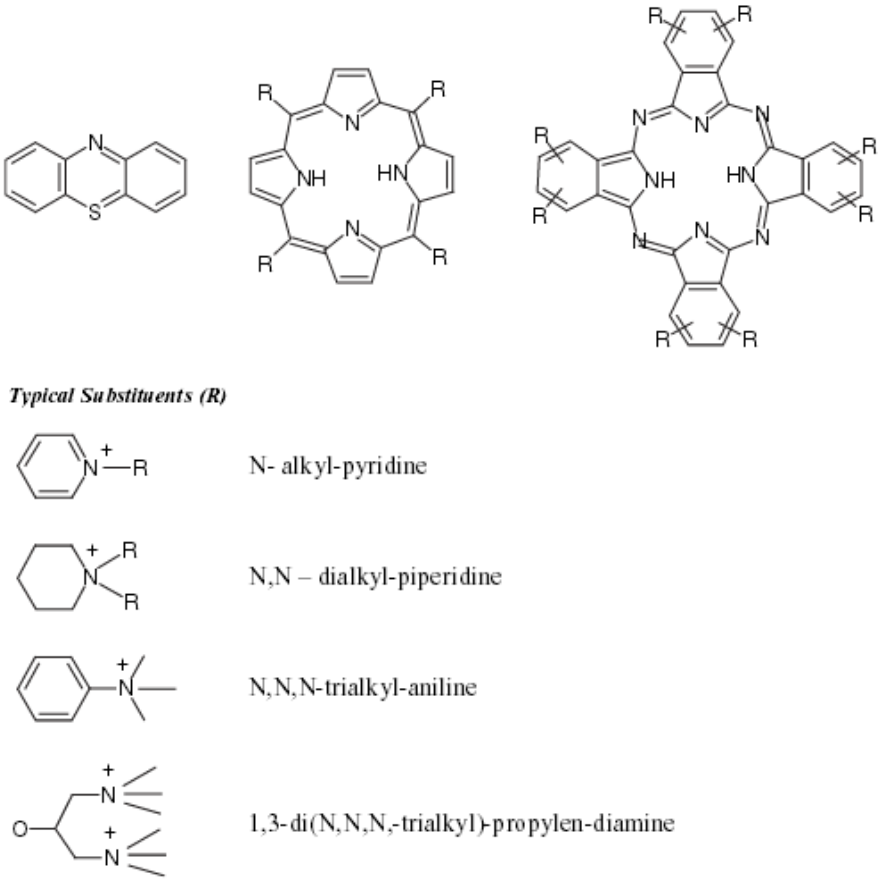

Figure 3 – Chemical representation of porphyrins with most typical substituents (Adapted from (Jori et al., 2006)

Due to their unique physic and chemical properties, porphyrins are used in many fields with innovative and exciting applications (Hamblin et al., 2011; Kadish et al., 2000; Bonnett, 1995), such as on nanomaterial's synthesis, in optical communications, in electro-optic dada processing and storage, photoionization processes, photo-inactivation in cancer, more recently to inactivate microorganisms and many studies are being done with promising new applications (Brown et al., 2004; Chou et al., 2000).

A study from 2004 has reported that a dicationic porphyrin and two tricationic porphyrins were powerful photosensitizing agents against *E.coli* (Débora Lazzeri et al., 2004). A recent study also concluded that similar compounds are remarkably successful in the lipid oxidation of *E.coli* (Lopes, 2013).

According to the literature, porphyrin derivatives can be used in the treatment of superficial cancers; topical treatment of dermatological problems, such as

psoriasis, acne, and Bowen's disease; gastrointestinal cancer; age related macular degeneration; cutaneous leishmaniasis; and viral infections, such as papillomatosis (Dai et al., 2009; Gardlo et al., 2003; M. Hamblin and Hasan, 2004; Josefsen and Boyle, 2012; O'Riordan et al., 2005; Sharman et al., 1999). Cationic porphyrins derivatives are also effective to inactivate *E.coli* (Spesia et al., 2005; Lopes, 2013).

1.2. Photodynamic inactivation of bacteria

Bacteria are a group of prokaryotic microorganisms, being the most abundant living organisms in nature. They can be found in almost all different environments in the planet, such as in hot springs, mammal intestines or in deep oceans (Darling, 2007). Bacteria have innumerous applications and benefits for humans, namely in food, pharmaceutic and clinic industries, among many others. Despite these facts, some bacteria are between the most dangerous pathogens for humans, other mammals, fish and plants. It is fundamental to find ways to inactivate properly and cost effective these microorganisms (Weller, 1988).

The produced ROS, by PDI, affects the integrity and functionality of proteins, lipids, nucleic acids and other molecules that are important components of bacterial cell membranes (Almeida et al., 2009; Stark, 2005). Among all nucleotides, guanine is the most sensitive to oxidation and has the lowest redox potential (Schulz et al., 2000)' (Agnez-Lima et al., 2012b). Oxygen singlet reacts with guanine base of DNA (Ravanat et al., 2000).

According to the Gram stain, there are two main bacteria divisions: Gram-positive and Gram-negative bacteria (Figure 4) (Jori and Brown, 2004b). They contrast in their outer surface composition, leading to different responses to antimicrobial agents (Malik et al., 1990). Gram positive bacteria can effortlessly interact with molecules such as PSs and can consequently be photoinactivated by the majority of PSs used in PDI (Malik et al., 1990; Bertoloni et al., 1992). However, Gram negative bacteria are usually impermeable to anionic drugs due to their highly negatively charged surface. The most used PSs developed for PDI, which are anionic drugs, are not effective alone against Gram negative

bacteria (Jori and Brown, 2004b). They are effective when administrated with a cationic agent (Kessel, 1992) capable to disrupt the cell wall enough to permit access of the PS (Jori and Brown, 2004b).

Several studies confirmed that there is a different susceptibility to photodynamic oxidation between gram-positive and gram-negative bacteria (Nitzan et al., 1989; M. Merchat et al., 1996; Minnock et al., 1996). Neutral and anionic PS were found to bind efficiently to gram-positive and to induce growth inhibition. Gram-positive bacteria do not have an outer cell membrane found in gram-negative, and the cell wall is high in peptidoglycan (Maisch et al., 2004; Tavares et al., 2010).). Gram-positive bacteria are characterized by the presence of a 40-80 nm thick outer peptidoglycan wall with no substantial amount of lipids and proteins. Contrarily, gram-negative bacteria contain an additional membrane layer, that is located outside the peptidoglycan layer and shows an asymmetric lipid structure composed by strongly negatively charged lipopolysaccharides (LPS), lipoproteins and proteins with porin proteins.

The enhanced susceptibility of Gram-positive cells to PDI is due to their thick outer wall constituted by more than a hundred peptidoglycan layers, closely associated with lipoteichoic and negatively charged teichuronic acids. Cell wall displays a relatively high degree of porosity, therefore, does not act as a permeability barrier for the most commonly used PS (Jori et al., 2006).

The same PS bind only to the outer membrane of gram-negative bacteria, being less efficient and showing a remarkable struggle to PDI (Bertoloni et al., 1992). The use of neutral and anionic PSs to inactivate gram-negative bacteria is only possible when combine with the use of membrane disorganizing substances, such as polymyxin B nonapeptide or Tris-EDTA (Nitzan et al., 1992

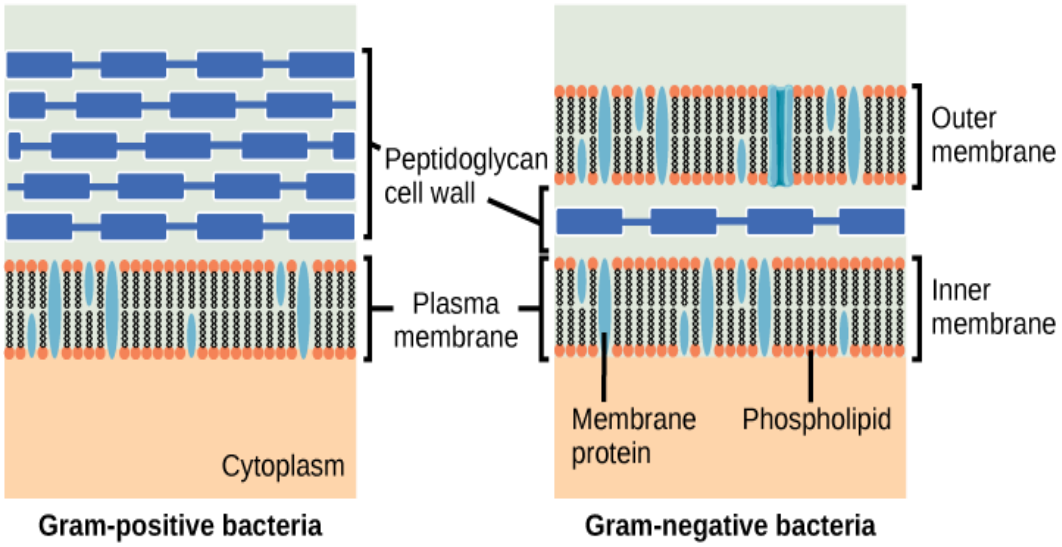

Figure 4 – Representation of Gram positive and Gram negative bacteria membranes. Gram-positive bacteria have a simpler membrane with peptidoglycan in the outer part. Gram-negative bacteria have an outer and inner membrane with a peptidoglycan wall between them - Source: http://cnx.org obtained at April 12 of 2014)

Gram model is very important in bacterial identification and characterization. However, some bacteria have complex variations from in this model, as for example *Aeromonas salmonicida* (Figure 5). This model allows obtaining a basic idea of the bacterial cell wall structure, but within the same type there are significant variations (Pereira et al., 2014; Romero et al., 1988).

Aeromonas salmonicida was discovered in Bavarian brown trout hatchery in 1894 by Emmerich and Weibel (Hiney and Olivier, 1999). It is a Gram negative bacterium that belongs to the *Proteobacteria* class. It has a regularly and well organized crystalline S-layer as its furthest cell wall component (Pereira et al., 2014). *Aeromonas salmonicida* is resistant to ampicillin and is susceptible to ciprofloxacin, gentamicin, amikacin and tobramycin (Dacanay et al., 2003).

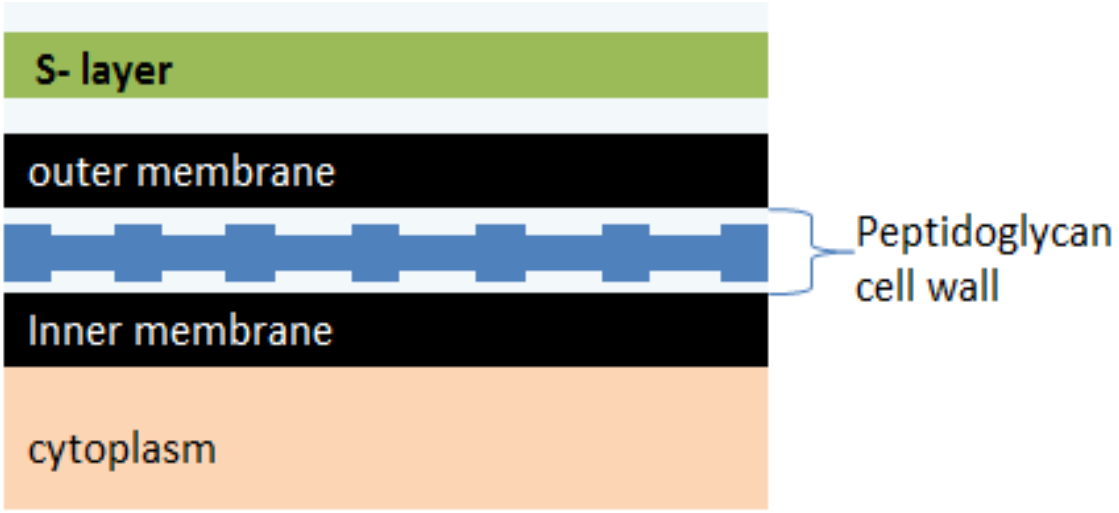

Figure 5 – Representation of *Aeromonas salmonicida* cell wall.

This bacterium is the etiological agent of furunculosis, a bacterial septicaemia of salmonids (Garduño et al., 2000) responsible for a noteworthy economic loss in the salmon farming industries (Dacanay et al., 2003). It is a facultative intracellular pathogen (Garduño et al., 2000), however the mechanisms by which this specie endures within macrophages are not totally understood and the S-layer is believed to be a central influence (Dacanay et al., 2003; Garduño et al., 2000).

It is a pathogenic bacterium for salmonids populations and other fish, in the natural environment but mostly when they grow in aquaculture (Almeida et al., 2009). The fish in aquaculture are constantly being thread by microbiological attacks (Dj, 1996). The problem is compounded by several factors, such as low microbial quality of water, i.e., high levels of faecal indicators of water quality; adverse and irregular environmental conditions, such as the rise in temperature, changes in salinity and in oxygen level, high concentrations, among others (Jori and Brown, 2004b).

PDI can be used in the environmental area, particularly for the decontamination of polluted waters. The traditional disinfection methods are very efficient and cover a high range of microorganisms; they involve the use of chlorine, chlorine dioxide, ozone and ultraviolet radiation, which makes it a high cost process with huge difficulties of implementation in large scales (Almeida et al., 2009).The S-layer of *A.salmonicida* is a two dimensional, paracrystalline tetragonal array of a single protein species, called A-proteins. A-proteins cover the complete

bacterial cell. The S-layer specifically binds to the O-antigen of *A. salmonicida* lipopolysaccharide (LPS). The S-layer interacts with outer membrane components underneath the O-polysaccharide layer. The S-layer forms a complex structure partially embedded in O-polysaccharide, which is essential for virulence. Because of its importance, S-layer had been subjected to structural, biochemical, genetic and functional studies (Garduño et al., 2000; Pereira et al., 2014). The S-layer has a crucial role in the early stages of infection, because at later stages it is eclipsed by the induction of a protective capsule that entirely covers the S-layer (Pereira et al., 2014). A study concluded that the S-layer is clearly involved in mediating high levels of adherence to non-phagocytic cells.).

1.3. Damage Mechanisms of Photodynamic Inactivation

Two mechanism of damage are proposed, according to literature, to explain how the damage is caused by PDI to microorganisms (Almeida et al., 2009; Moan et al., 1979, M. R. Hamblin and Hasan, 2004; Ito, 1978; Lopes, 2013; Arrojado et al., 2011; Bertoloni et al., 2000). In both gram negative and gram positive bacteria it was demonstrated the disappearance of the plasmid super coiled fraction (Fiel et al., 1981).

The process of light absorption and energy transfer are the basis of PDT. The ground state PS has two electrons with opposite spins, known as singlet state, in the lowest energy molecular orbital. After the absorption of photons, one of the unpaired electrons is enhanced into a higher energy orbital without misplacing it spin, known as the first excited singlet state. Once in the first excited singlet level, the PS may endure the process known as intersystem crossing whereby the spin of the excited electron inverts to form the triplet state with parallel spins with a longer lifetime than the previous state. The PS may also lose energy by emitting light, fluorescence, or by internal conversion into heat (Figure 6) (Ito, 1978; Castano et al., 2004).

There are two types of reactions where the excited triplet can be submitted: Type I and Type II. Both reaction types can occur simultaneously and the ratio between these processes depends on the type of PS used, the concentrations

of substrate and oxygen concentration (Agnez-Lima et al., 2012b; Girotti, 2001). Regarding the Type I reaction, the excited triplet PS can react directly with a substrate, such as the cell membrane, and transfer a proton or an electron to form a radical anion or a radical cation (Ma and Jiang, 2001). The formed radicals may react with oxygen and produce oxygen reactive species. The pathways of this reaction often involve initial production of superoxide anion by electron transfer from the triplet PS to molecular oxygen (Bilski et al., 1993). In the Type II reaction, the triple PS can transfer its energy directly to molecular oxygen, which is a triplet in the ground state, and form excited singlet oxygen (Castano et al., 2004). The resulting products of both type mechanisms may cause damage to biomolecules. The photo-inactivation process is not yet completely acknowledged, well as the extent of bacterial damage cause by this therapy. There are some studies following a proteomic approach that proved induced damage into proteins and DNA by PDI (Moan et al., 1979; Rapp et al., 1973; Felber TD et al., 1973); however, very few have studied the effects of PDI on membrane lipids Reactive oxygen species can induce cell damage by the following ways: increasing ion permeability [Na^+/K^+] leakage, loss of repair facility, lysis, inhibition of respiration, inhibition of ribosome assembly, inhibition of replication, base substitution and strand breakage (Wainwright, 1998).

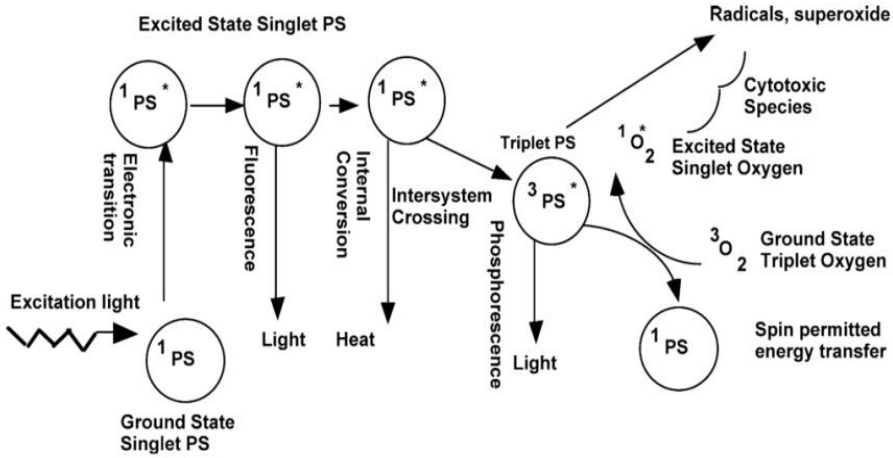

Figure 6 – Photo-physic and photo-chemical representation of the photoinactivation process – Adapted from (Castano et al., 2004)

Over time, many studies have demonstrated that bacteria have been killed and inactivated by the use of different combinations of PS and light (Pereira et al., 2014; Tavares et al., 2010; Alves et al., 2009a) However, the mechanism of

bacterial inactivation has not yet been fully elucidated (Castano et al., 2004). In the 90's, it was reported that there are fundamental differences in susceptibility of PDI between Gram positive and Gram negative bacteria (M. R. Hamblin and Hasan, 2004; Maisch et al., 2004; Demidova and Hamblin, 2005).

1.3.1 Type 1 Mechanism

According to the Type 1 mechanism, electrons or protons from the reducing agent (RH) are transferred to PS on the excited triplet state (^3S) (Apel and Hirt, 2004). This transference, results in the formation of free radicals, the radical anion of the PS ($S^{-\bullet}$), and the substrate radical ($R^{\bullet}$). By a peroxidation chain reaction process, both radicals can induce the formation of a lipid radical ($L^{\bullet}$). The radical substrate specie, $R^{\bullet}$, reacts with an oxygen molecule and leads to the formation cytotoxic species ($ROO^{\bullet}$). Radical hydroperoxides, superoxide radicals and hydrogen peroxide are part of the cytotoxic species formed (Huang et al., 2012; Girotti, 2001).
This processed can be summarized, by way of:

1) $^3S + RH \rightarrow S^{-\bullet} + R^{\bullet} + H^+$
2) $R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$

The substrate radical has sufficient oxidative power to originate hydroxyl radicals, $OH^{\bullet}$ (Kappus and Sies, 1981). The originated compounds have a short life time, so they are highly reactive. The radical anion from the PS can also initiate the reduction of a sequential electron from O_2 to H_2O_2. The H_2O_2 is reduced in presence of Fe^{2+}, according to a reaction called Fenton.

3) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$

Besides the production of hydroxyl radicals, superoxide radicals are also produced by the Type 1 mechanism. However, these toxic species are not particularly reactive in biological systems, unless when suffer protonation ($HOO^{\bullet}$) leading to the production of H_2O_2 and O_2.

4) $2HOO^{\bullet} \rightarrow H_2O_2 + O_2$

The formed $OH^{\bullet}$ triggers a chain peroxidation chain that is initiated by the abstraction of one allyl hydrogen from an unsaturated lipid, LH. In phospholipids, the abstracted hydrogen is usually provided by fatty acids in the sn-2 position. This reaction originates a radical lipid, $L^{\bullet}$ that reacts with $^3O_2$ to produce a lipid peroxyl radical, $LOO^{\bullet}$. Through chain reactions, $LOO^{\bullet}$ is reduced into lipid hydroperoxide, LOOH (Jiang et al., 1992):

5) $LH + HO^{\bullet} \rightarrow L^{\bullet} + H_2O$
6) $L^{\bullet} + {}^3O_2 \rightarrow LOO^{\bullet}$
7) $LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$

1.3.2. Type 2 Mechanism

In the oxidation mechanisms of Type 2, it is produced singlet oxygen, exceptionally highly reactive specie with the ability to oxidize biomolecules.
A PS, when irradiated on an appropriate wavelength, is excited, and passes to it triplet state, ^3S. In this state, the PS transfers energy to the molecular oxygen on the fundamental state ($^3O_2$). This promotes the reversal of the spin in one of the electrons of the triplet oxygen, converting it into singlet oxygen ($^1O_2$). The singlet oxygen may also be formed during the degradation of lipid peroxides.

1) $^3S + {}^3O_2 \rightarrow S0 + {}^1O_2$

Singlet oxygen can react directly with unsaturated fatty acids of phospholipids originating hydroperoxides, with the double bond relocated to the allyl position.

2) $LH + {}^1O_2 \rightarrow LOOH$

The formed lipid hydroperoxides, in the presence of metallic ions, may react as on Mechanism 1 originating a chain reaction. With absence of these ions,

LOOH will accumulate proportionally with the light dosage (Jiang et al., 1992; Lopes, 2013).

2. Bacteria membrane lipids

Lipids are organic compounds and the majority contains fatty acids chains. These compounds are the foundation for structure and function in membranes of living cells. Lipids are non-polar, so they are soluble in nonpolar environments consequently not being soluble in water. Biological membranes are composed of lipid bilayers that act as a boundary to various cellular structures; however they also allow for careful transfer of ions and organic molecules into and out of the cell (Porter et al., 1995; Baker et al., 1941). Cell membrane is organized as a lipid bilayer, a polar membrane made of two layers of lipid molecules. The membrane is arranged with the hydrophilic phosphate heads pointing out on either side of the bilayer and the hydrophobic tails are pointed to the core of the bilayer. Phospholipids are divided in two main groups, glycerophospholipids and sphingomyelins. Phospholipids are a class of lipids that are the most abundant molecules in all cellular membranes and are responsible for bilayer form on these membranes (Figure 7). Phospholipids consist of a glycerol molecule and two fatty acids, unlike triglycerides, which have three fatty acids (Igene et al., 1980), and a phosphate group that can be linked to charged or polar chemical groups (Dowling et al., 1986).

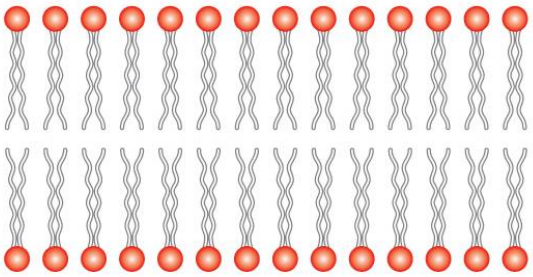

Figure 7 – Phospholipids disposition in biological membranes, with the hydrophobic tails disposed in the center and the hydrophobic heads disposed in the external part of the membrane – Source: https://lh3.ggpht.com/

Fatty acids are carboxylic acids with an aliphatic chain, that are divided into saturated, monosaturated and polysaturated acids, according to the presence and number of double bonds in the chain(de Geus et al., 2001). The main fatty acids present in the membrane of *Aeromonas salmonicida* are C16:1, C16:0 and C18:1 (Lambert et al., 1983).

The phosphate group the negatively – charged in the polar head, which is hydrophilic. The fatty acids chains are uncharged, nonpolar tails, which are hydrophobic. A phospholipid is an amphipathic molecule because it has hydrophilic and hydrophobic parts (Antonny et al., 1997). Some lipid tails consist of saturated fatty acids and some contain unsaturated fatty acids (Figure 8), giving fluidity to the tails that are constantly in motion (Zhang and Rock, 2008; Baker et al., 1941).

Singer and Nicolson proposed, in 1972, a model to explain how molecular components are displayed in biological membranes. According to the proposed model, membrane lipids are homogenous distributed in the membrane (Singer and Nicolson, 1972). Fluid mosaic model was important to explain the mechanisms that occur in biological membranes, however, new experimental data, demonstrate that cell membranes tend to be polarized laterally because of polar chemo-receptors proteins, such as proteins that act in the actin polymerization and proteins involved in cell division (Shapiro et al., 2002). The biological membranes are constituted of proteins and lipids that create permeability barriers for cells. The main function of the membrane is to separate the inner content of the cell from the external environment. These membranes perform as selective permeability barriers, they allow the directional transport of some molecules, but they exclude the passage of others. Some present lipids act as second messengers, transmitting signals that potently influence important regulatory functions, such as the cell division and cell death (Vance, 2001). The diversity of phospholipids and the asymmetric outlook in the membrane bilayer; and the physic and chemical properties of each phospholipid group, led to a new model where the lipid disposal is heterogeneous with a formation of membrane micro-domains (Vereb et al., 2003). This disposal of lipids explains the lateral polarization on cell membranes (Matsumoto et al., 2006). Some studies had reported that the lateral heterogeneity in lipid

domains, occur in prokaryotes and eukaryotes (Dowling et al., 1986; Vereb et al., 2003).

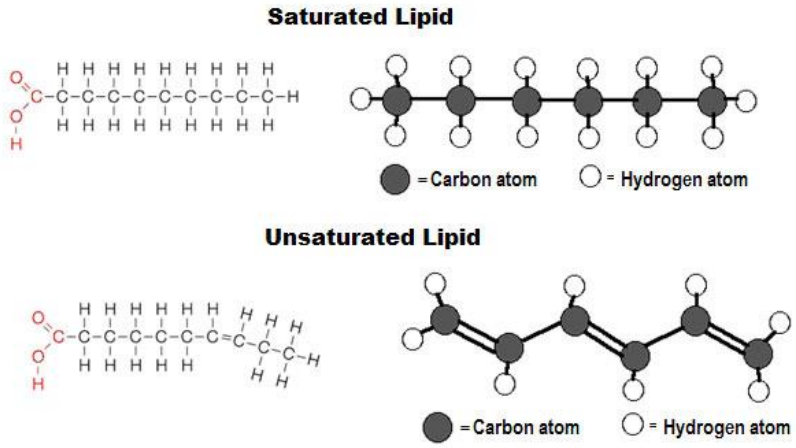

Figure 8 – Representation of unsaturated and saturated fatty acids. The double bond is represented in unsaturated fatty acids. Source: http://00.edu-cdn.com.

The most abundant membrane lipids in all living organisms, from bacteria to mammalian, are the glycerophospholipids. The most relevant classes of these compounds are phosphatidylcholine, phosphatidylethanolamine, fosfatidilinosinol, phosphatidylglycerol and phosphatidylserine (Vance and Vance, 2002)

The structure of glycerophospholipids consists of a molecule that contains hydrophobic fatty acyl chains esterified to both *sn1* and *sn2* positions of glycerol (Vance, 2001).

In prokaryotes, phospholipids are synthetized by the same reactions as in mammalians and plants, with the difference that the vast majority of bacteria do not make phosphatidylcholine. The main phospholipid constituents of bacteria membrane are phosphatidylethanolamine, more than 75 per cent, and phosphatidylglycerol, with smaller amounts of cardiolipin and phosphatidylserine (Zhang and Rock, 2008). The phosphatidic acid consists of glycerol-3-phosphate in which the positions C1 and C3 are esterified with two fatty acids. This compound is the simplest glycerophospholipid and the precursor for all the other glycerophospholipids. The fatty acids found on this

sub-group have 16 to 20 carbons. Usually, the C1 position is occupied by a saturated fatty acid and the position C2 by one unsaturated fatty acid.

3. Aims of the study

Our research group is pioneer in the study of membrane lipids after PDI. Studies conduct by the group established an irrevocable prove that membrane lipids are important cell targets of PDI and the effectiveness of this therapy is highly dependent on the photosensitizer choice. The group has focused the research on cationic porphyrins acting as photosensitizers. The present work arises as a continuation of studies that have been developed by the group, studying a new bacterium.

The aims of the current study are:

1) Evaluate the effect of PDI on membrane lipids of *Aeromonas salmonicida* using different cationic porphyrins with different charge distribution as PSs in different times of visible light exposure. To evaluate this effect it was used the FOX II method to quantify the lipid hydroperoxides and the analysis of the fatty acids profile variations by gas chromatography in each irradiation time for all the PSs. The aim has been achieved using PSs with different positive charge distribution, in different times of light exposure.
   A.salmonicida was chosen because it presents complex and different properties on the cell wall and the photo oxidation process is not well understood.

2) Evaluate the effect of photodynamic inactivation with different porphyrins, as photosensitizers, on the cell viability of *Aeromonas salmonicida* by classical methods of microbiology; and establish a relation between the lipid oxidation with the cell survival

Chapter II

Methods

1. Photosensitizers

For the current study were used the following porphyrins, as PSs: 5,10,15–tris(pentafluorophenyl)–20–(1 methylpiridinum-4-yl) porphyrin iodide (Mono-Py^{+}-Me-PF); 5,15-bis(1-methylpyridinium-4-yl)-10,20-bis(pentafluorophenyl) porphyrin diiodide (Di-Py^{+}-Me-Di-PF_{opp}); 5-(pentafluorophenyl)-10,15,20-tris(1-methylpiridinum-4-yl) porphyrin triiodide (Tri-Py^{+}-Me-PF); 5,10,15,20-tetrakis(1-methylpiridinium-4-yl) porphyrin tetra-iodide (Tetra-Py^{+}Me). The chemical structures of the used porphyrins are represented on Figure 9.

The porphyrins used in this work were prepared in two steps according to the literature (Tomé et al., 2004). The neutral porphyrins were synthesised by the crossed Rothemund reactions using pyrrole and the adequate benzaldheydes at reflux in acetic acid and nitrobenzene. The resulting porphyrins were separated by column chromatography and pyridyl groups' quaternized by reaction with methyl iodide. Porphyrins were purified by crystallization from chloroform/methanol/petroleum ether and their purities were confirmed by thin layer chromatography and by ^{1}H NMR spectroscopy.

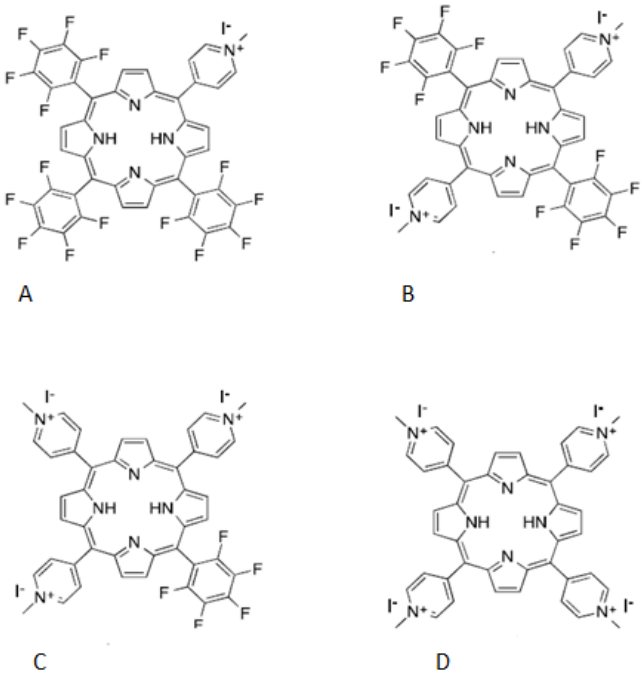

Figure 9 – Chemical representation of the used porphyrins - A - Mono-Py+-Me; B - Di-Py+-Me, C - Tri-Py+-Me-PF, D - Tetra-Py+-Me

2. Bacterial Growth conditions

The growth conditions were the same in all tests to ensure reproducibility and aseptic conditions were always assured.

Aeromonas salmonicida strains were kept at 4ºC in a solid agar medium, TSA (Triptic Soy Agar). Bacterial strains from fresh cultured plates were inoculated in 10 mL of liquid medium, Trypticase Soya Broth (TSB) and grown aerobically under 1300 rpm at 30ºC.

An aliquot was transferred twice into new fresh medium and grown for 18 and 24 hours, respectively, in the same conditions to reach the initial stationary phase with an optic density of ≈1.6 at 600nm (Standard error ± 0,1).

3. Bacterial preparation

Bacterial cultures were concentrated and lysed from the 180 mL liquid culture by centrifuging at 4000 rpm for 10 minutes with a temperature of 4ºC. The supernatant liquid was removed, remaining the pellet only. The pellet was consecutively washed with phosphate buffered saline solution (PBS) and centrifuged according to the preview conditions. The remaining pellet was resuspended in 15 mL of PBS solution.

4. Irradiation assay

The diluted bacterial suspensions were distributed in glass beakers, with a final volume of 60 mL per beaker, according to the Table II. The beakers were incubated in the absence of light with 5.0 µM of porphyrin for 15 minutes under 100 rpm stirring at room temperature, in order to promote the PS binding to cells. The irradiation by light was made using an illumination system designed by 13 parallel OSRAM 21 lamps, each one with 18 W, with an irradiance of 40

W m^{-2}. The light was emitted in the range of 380-700 nm. Bacterial suspensions were irradiated up to 270 minutes and subsamples were collected at the start of the irradiation and after 90 and 270 minutes of light exposure. After each period, the cells were washed in PBS and diluted into Milli-Q water. The samples were sonicated six times within 1 minute, interposed with 1 minute left in the ice. The cell lysis was also endorsed using the FastPrep protocol with glass beads 4 times and kept in methanol overnight. Control samples were incorporated in all PDI experiments, light control and dark control.

Table II - Distribution of the irradiated glasses, Light Control; Dark Control; and Sample.

	Bacterial Extract	**PBS Solution**	**Porphyrin** (5.0 µM)
Light Control	5mL	55mL	0
Dark Control	5mL	54.4mL	600µl
Sample	5mL	54.4mL	600µl

The lipids were extracted according to Matyash, 2008 method – Lipid extraction by methyl-*terc*-butyl ether (MTBE) for high throughput lipidomics. It was added methanol to the samples and left overnight. MTBE was added and the separated organic phase was collected. The procedure was made a second time to assure an higher separation with a solution of MTBE, methanol and water (10:3:2.5, v/v/v). The collected samples were dried in vials with nitrogen steam and kept at -20 ºC.

5. Phospholipids quantification

The quantification of phospholipids was made adding perchloric acid (0.5 mL, 70% m/v) to the lipid dry extracts. The mixture was incubated for 60 min at 180 ºC on a block heater (*Block Heater SBH200D/3, Stuart®, Bibbly Scientific Ltd., Stone, UK*) to cleave the phosphate head. After the incubation period, was added 3.3 mL of Milli-Q water, 0.5 mL of ammonium heptamolybdate (2.5 %) and 0.5 mL of ascorbic acid (10 %) with a strong stirring between the additions. The combination was incubated for 10 min, at 100ºC on a heat bath (*Precisterm, JP Selecta S.A., Barcelona, Spain*). At the same time, it was prepared phosphate standards with concentrations from 0.1 up to 2 µg mL^{-1} with $NaH_2PO_4.2H_2O$ (100 µg mL^{-1}), receiving the same treatment with the only exception of the block heater. The absorbance was measured, at a wavelength of 800 nm at room temperature, in a spectrophotometer UV-Vis (*Multiskan GO, Thermo Scientific, Hudson, NH, USA*). The quantification of phosphate was calculated by a linear regression and the phospholipid content was determinate multiplying the phosphate by 25.

6. Quantification of the Lipid Hydroperoxides

The quantification of the lipid hydroperoxides was made by the FOX 2 method (ferrous oxidation-xylenol orange). The quantification was made using 50 µL of

lipid extract and 950 µL of FOX 2 reagent. At the same time it was made the standards with H_2O_2 from 0 to 1.2 mM (H_2O_2 1 mM, FOX 2 reagent and Milli-Q water). The mixtures were homogenized and left 30 min in dark in order to occur the oxidation reaction of Fe^{2+} in Fe^{3+}. Fe^{3+} was detected by the presence of xylenol. The absorbance was measured in a wavelength of 560 nm in a spectrophotometer UV-Vis (*Multiskan GO, Thermo Scientific, Hudson, NH, USA*) and the quantity of lipid hydroperoxides was calculated by linear regression.

7. Gas Chromatography (GC-MS) Analysis

Approximately 40 µg of phospholipid were converted in methyl esters, adding hexane (1mL) to the extract and a solution of KOH (200 µL, 2.0 M) in methanol. The samples were well homogenized in vortex for 3 min. It was added a saturated solution of sodium chlorite (NaCl 2 mL) and homogenized in vortex for 2 min, followed by a 2000 rpm centrifugation for 5 min. The organic phase was collected for a new tube and dried in speed vac. It was added 19µL of hexane and it was injected 5 µl in GC. For the identification of fatty acids the correspondent methyl esters were analysed by gas chromatography-mass spectrometry (GC-MS) on an Agilent Technologies 6890N Network (Santa Clara, CA) equipped with a DB-1 column with 30 m of length, 0.25 mm of internal diameter, and 0.1 µm of film thickness (J&W Scientific, Folsom, CA). The GC was connected to an Agilent 5973 Network Mass Selective Detector operating with an electron impact mode at 70 eV and scanning the range m/z 40-500 in a 1 s cycle in a full scan mode acquisition. The oven temperature was programmed from an initial temperature of 40 ºC, standing at this temperature for 0.5 min, a linear increase to 220 ºC at 20 ºC/min, followed by linear increase at 2ºC/min to 240ºC, and then at 5 ºC/min to 250 ºC. The injector and detector temperatures were 220 and 230 °C, respectively. Helium was used as carrier gas at a flow rate of 1.7 mL/min.

8. Gas Chromatography (GC-FID) Analysis

Approximately 40 µg of phospholipid were converted in methyl esters, adding hexane (1mL) to the extract and a solution of KOH (200 µL, 2.0 M) in methanol. The samples were well homogenized in vortex for 3 min. It was added a saturated solution of sodium chlorite (NaCl 2 mL) and homogenized in vortex for 2 min, followed by a 2000 rpm centrifugation for 5 min. The organic phase was collected for a new tube and dried in speed vac. It was added 19µL of hexane and it was injected 5 µl in GC.

Methyl esters of fatty acids were analysed on a PerkinElmer Clarus 400 gas chromatograph (Waltham, MA) equipped with a flame ionization detector (FID) and a DB-1 column with 30 m of length, 0.25 mm of internal diameter, and 0.1 µm of film thickness (J&W Scientific, Folsom, CA). The oven temperature program used was 40 ºC. The injector and detector temperatures were 220 and 230 °C, respectively. Hydrogen was used as carrier gas at a flow rate of 1.7 mL/min.

9. Viability texts

Bacterial cultures were growth to correspond to the early stationary phase. Cultures were diluted in PBS to a final concentration of ≈ 10^9 CFU mL^{-1} and distributed in beakers with a volume of 10 mL per beaker. Beakers were incubated in the dark with porphyrin for 10 min at room temperature under 100 rpm stirring to promote the porphyrin binding to cells. After this 10 min in darkness the beakers were irradiated by a light system, formed of 13 parallel OSRAM 18 W/21–840 lamps with an irradiance of 4.0 mW cm^{-2}, emitting in the range of 380-700 nm. The suspensions were irradiated up to 270 min with 5.0 µM of each PS. Samples of 100µL were collected before irradiation (Time 0), after 60, 90, 180 and 270 min of light exposure

After each photosensitization period, the cells were diluted in PBS, plated in TSA and incubated at 37 ºC for approximately 24 hours. The cell viability was determined by counting the CFU of each sample at the most appropriate dilution on the agar plates.

Control samples were carried out simultaneously with the PDI procedure: light control comprised a bacterial suspension exposed to light; and dark control comprised by a bacterial suspension incubated with PS at the studied concentrations but protected from light.

10. Statistical Analysis

Statistical analyses were performed using GraphPad® Prism 5. Normal distributions were assessed by the same software. The significance of all porphyrins and irradiation time on bacterial was assessed by two-way ANOVA analysis of variance. The Bonferroni test was executed and a value of $p < 0.05$ was considered significant.

Chapter III

Results

1. Quantification of Lipid Hydroperoxides

Lipid hydroperoxides (LOOH) were quantified according to the FOX II method, via oxidation of Fe^{2+} by xylenol orange, which is a simple and fast technique to evaluate the oxidative effect in *A. salmonicida* membrane phospholipids.

This method allows quantifying the lipid hydroperoxides on the total lipid extract, based on the oxidation of Fe^{2+} into Fe^{3+}. Oxidation occurs in the presence of hydroperoxides in acidic conditions. Ferric ions (Fe^{3+}) react with xylenol orange, originating a violet-blue complex, with a maximal absorbance of 550-600nm. The following reaction summaries this process:

1) $Fe^{2} + LOOH \rightarrow Fe^{3+} + OH^{\bullet}$
2) Fe^{3+} + Xylenol Orange → Violet-Blue Complex (560nm)

The ferric ions complex was measured by UV absorbance at 560 nm in a microplate reader.

The method was used to evaluate the photo-oxidative effect on *Aeromonas salmonicida* cell membrane, with the different PSs in the same conditions. It was measured in three times of irradiation, the time zero (0), time ninety (90) and two hundred and seventy (270) minutes of irradiation. The tests were performed separately and in different days to ensure their reliability and reproducibility. On Figure 10 are presented the results of lipid hydroperoxides quantification by FOX II method. All the samples in the different times are compared with the light control (sample submitted to light exposure and without PS)

The amount of lipid hydroperoxides on light control is lower than in the irradiated samples, being a baseline for natural oxidation along the time.

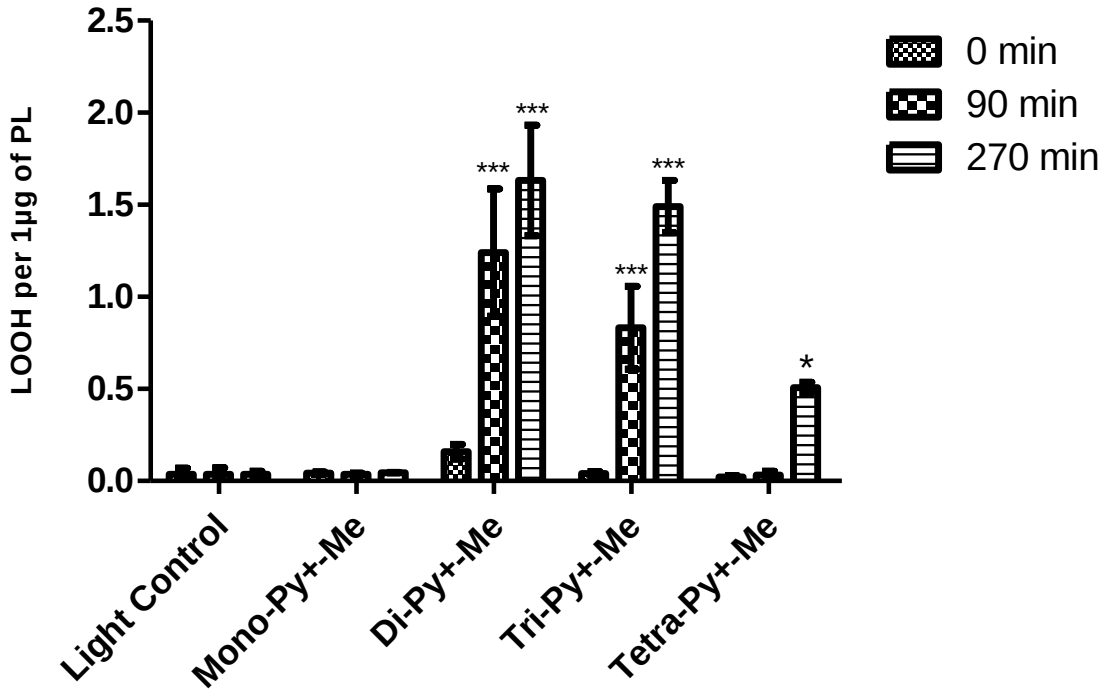

Figure 10 – Lipid hydroperoxides of *Aeromonas salmonicida* quantification by FOX II after PDI, in 0, 90 and 270 minutes of light exposure. results with the porphyrins: Mono-Py^{+}-Me-PF; Di-Py^{+}-Me-PF opp.; Tri-Py^{+}-Me-PF; and Tetra-Py^{+}-Me-PF. The present values are the averages with ± standard deviation. It was effected an one-way ANOVA with significant difference with Light Control ***, $P<0.001$; * $P < 0.05$.

After the irradiation periods, both 90 and 270 minutes, an increase in LOOH indicates an oxidative occurrence on *Aeromonas salmonicida* lipids.

At 90 minutes of light exposure, the highest quantities of LOOH were obtained using the porphyrin Di-Py^{+}-Me-PF with an average of approximately 1.2 LOOH per µg of phospholipid. High LOOH quantities were also obtained using Tri-Py^{+}-Me after the same irradiation period with an average of approximately 0.8 LOOH per µg of phospholipid. Mono-Py^{+}-Me and Tetra-Py^{+}-Me were the PSs that showed the lowest levels of lipid hydroperoxides, with insignificant variances over this period of time.

At 270 minutes of light exposure the lipid hydroperoxides quantification shows, once again, higher quantities in the assays using Di-Py^{+}-Me and Tri-Py^{+}-Me, with an approximately average of 1.6 and 1.5 LOOH per µg of phospholipid,

respectively. The lowest measures were obtained when it was used Tetra-Py^+-Me and Mono-Py^+-Me as PSs. Mono-Py^+-Me did not the led to any significant production of LOOH and Tetra-Py^+-Me led to a production of approximately 0.6 LOOH per µg of phospholipid.

According to the formation of LOOH, one can claim that, after 90 minutes of light exposure, the PSs Di-Py^+-Me was the most effective, followed by Tri-Py^+-Me. Mono-Py^+-Me and Tetra-Py^+-Me were the PSs with the lowest efficiency after 90 minutes of irradiation. Similar results were obtained after 270 minutes of irradiation.

Regarding these results, it is possible to establish an order of effectiveness for both irradiation times, after 90 and 270 minutes of light exposure, according to the formation of LOOH. The establish order is summarized on Table III to better compare the two intervals of time.

Table III – Established order of effectiveness for each porphyrin, as PS, after 90 and 270 minutes of exposure to light source. The order was done taking in account the formation of LOOH, measured by the FOX II protocol.

Order	90 minutes	270 minutes
1	Di-Py^+-Me	Di-Py^+-Me
2	Tri-Py^+-Me	Tri-Py^+-Me
3	Tetra-Py^+-Me	Tetra-Py^+-Me
4	Mono-Py^+-Me	Mono-Py^+-Me

The production of LOOH was very high using Di-Py^+-Me and Tri-Py^+-Me after 270 minutes of exposure to light. As seen on Table III, the established order does not vary in both irradiation periods, which means that the efficiency of the PSs is continuous over the irradiation period.

2. Identification of Fatty Acids by GC-MS

The fatty acids profiles of *Aeromonas salmocida* lipid extracts were obtained using Gas Chromatography with Mass Spectometry (GC-MS). According to this methodology, it was indified the following fatty acids: C14:0; C14:0 methyl; C15:0; C16:1; C16:0; C18:1n9; C18:1n13; and C18:0. The identified fatty acids represent the most abudant ones in *Aeromonas salmonicida* cell wall. All the fatty acids were identifed with a simillarity over 95% to the digital library and are in accordance with the literature (Dacanay et al., 2003).

3. Relative Quantification of Fatty Acids by GC-FID

The relative abudance of fatty acids was calculated over the analysis of chromatograms obained by Gas Chromatography – Flame ionization detector (GC-FID). It gives the information of the fatty acids profile for the different conditions. The sotware allows to integrate the areas of each peak mannually in order to calculate the relative abudance of each fatty acid. An example of chromatogram for each PS for the three irradiation times is presented on the next figures.

In the assay using Mono-Py^{+}-Me, as PS, there is no significant alteration in fatty acids profile along the irradiation time (Figure 11).

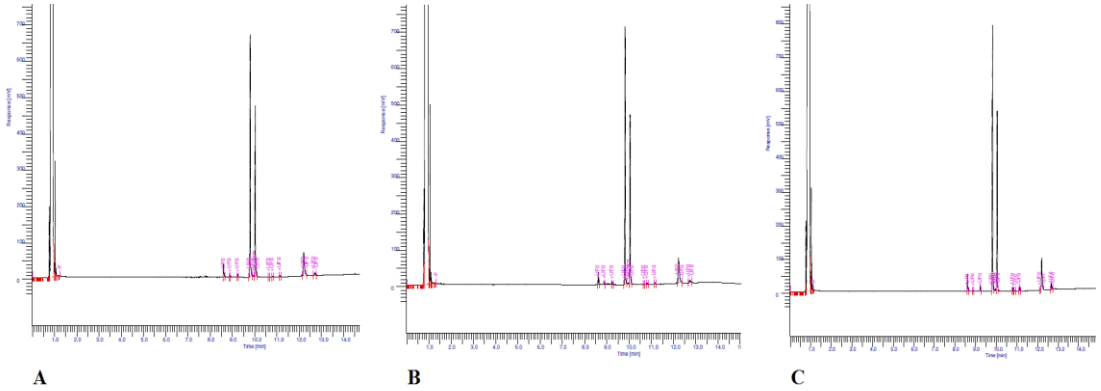

Figure 11 – Chromatograms obtained by GC-FID for Mono-Py^{+}-Me using the software TotalChrome. A – Chromatogram for 0 min of irradiation; B - Chromatogram for 90 min of irradiation; Chromatogram for 270 min of irradiation.

In the assay using Di-Py^{+}-Me, as PS, there is a remarkable alteration in fatty acids profile along the irradiation time, as it is possible to observe on Figure 12.

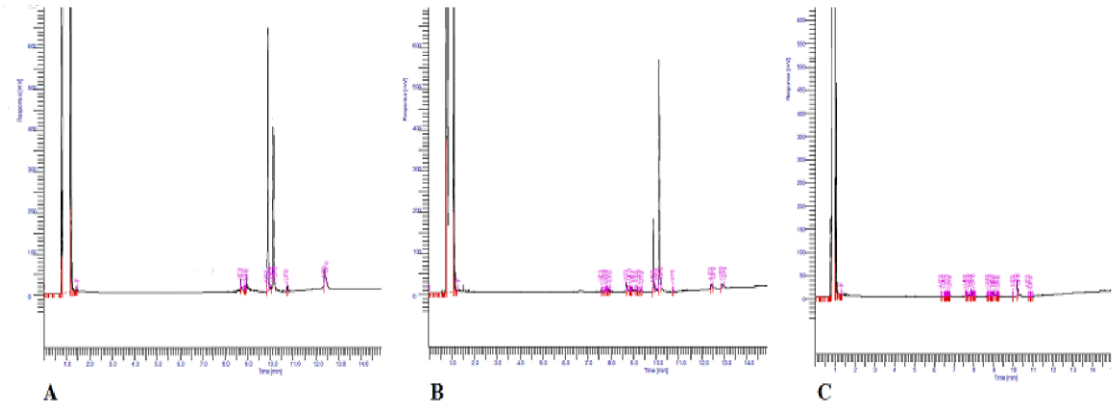

Figure 12 - Chromatograms obtained by GC-FID for Di-Py^{+}-Me-PFopp using the software TotalChrome. A – Chromatogram for 0 min of irradiation; B - Chromatogram for 90 min of irradiation; Chromatogram for 270 min of irradiation.

In the assay using Tri-Py^{+}-Me-PF, as PS, the alteration in fatty acids profile along the irradiation time is also notable. The profile is presented on Figure 13.

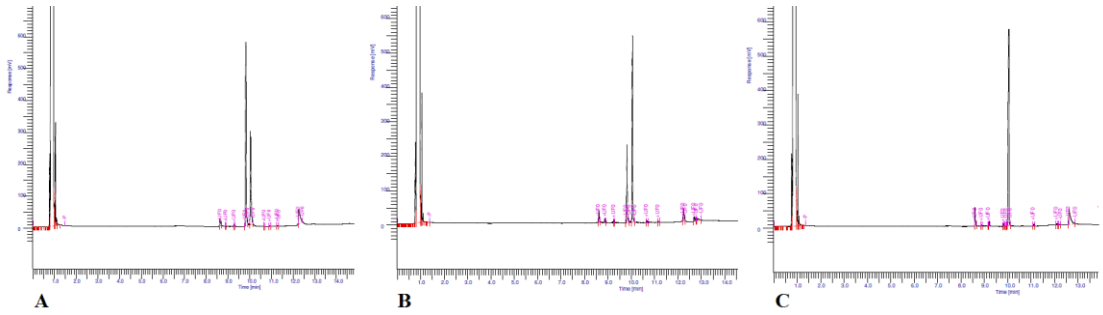

Figure 13 - Chromatograms obtained by GC-FID for Tri-Py^{+}-Me-PF using the software TotalChrome. A – Chromatogram for 0 min of irradiation; B - Chromatogram for 90 min of irradiation; Chromatogram for 270 min of irradiation.

In the assay using Tetra-Py^{+}-Me-PF, as PS, the alteration in fatty acids profile along the irradiation time is also verified; however, it seems smaller than in the previous PS. The profile is presented on Figure 14.

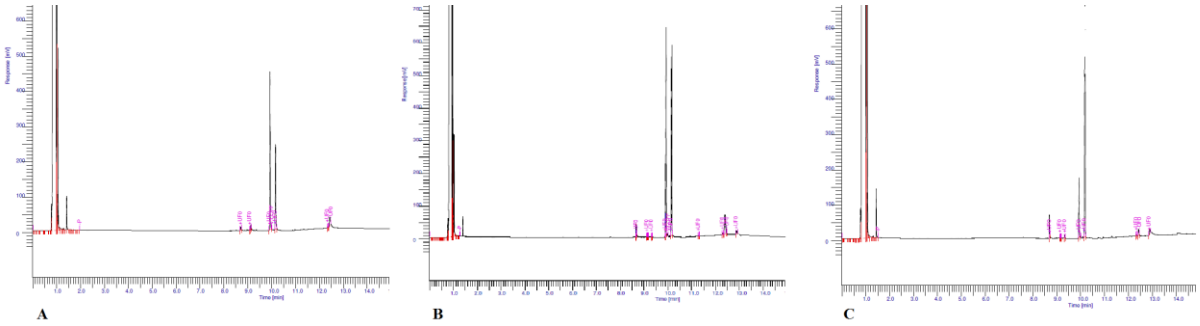

Figure 14 - Chromatograms obtained by GC-FID for Tetra-Py^{+}-Me-PF using the software TotalChrome. A – Chromatogram for 0 min of irradiation; B - Chromatogram for 90 min of irradiation; Chromatogram for 270 min of irradiation.

The presented chromotagrams give a fast and easy idea on how the fatty acids profile differs over the irradiation time. However, this method does not give an

objective quantification. The relative quantification was calculated by integration of the peaks areas in each fatty acid and the percentage was calculated individually for each data sample. Figure 15 shows the relative abudance of each fatty acid for the four studied PSs.

The analysis of relative abudance for each fatty acid provide coerent and reprodutible results that represent the same fatty acids profile reported in chromatograms. Using these results it is possible to analyze the influence of each porphyrin in the oxidation of membrane lipids.

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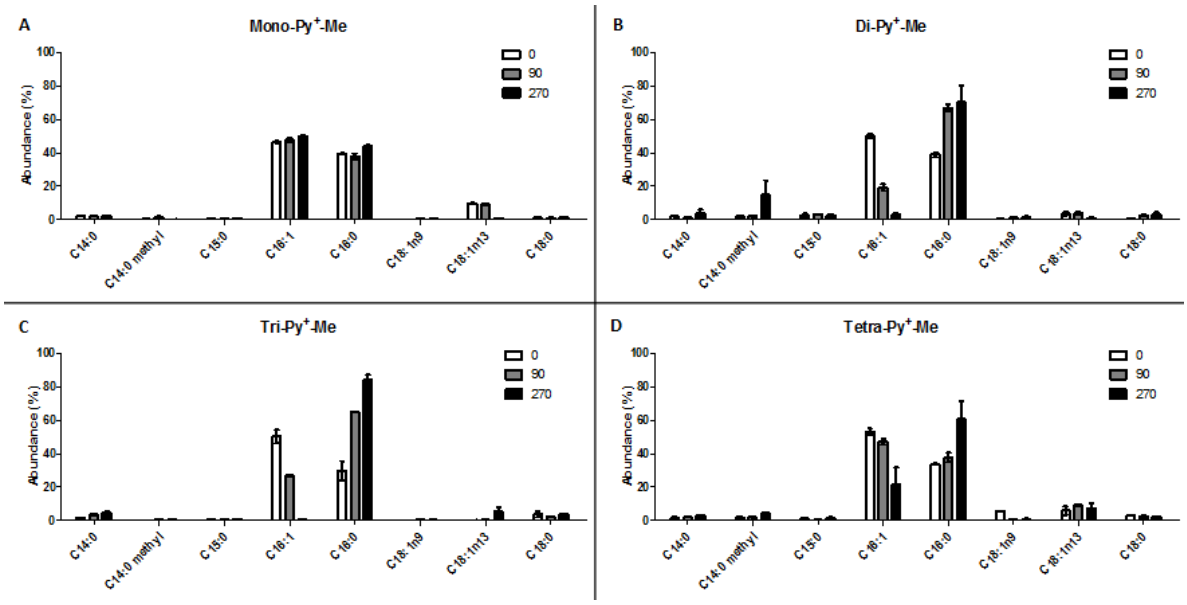

Figure 15 – Relative abudance (%) of each fatty acid during the three irradiation times. A – Relative abudance using Mono-Py^{+}-Me-PF; B - Relative abudance using Di-Py^{+}-Me; C - Relative abudance using Tri-Py^{+}-Me; D - Relative abudance using Tetra-Py^{+}-Me. The present values are the averages with ± standard deviation.

The assays using Mono-Py^{+}-Me do not demonstrate significant variances in the fatty acids profile during the exposure time. Except in this assay, using Mono-Py+-Me as PS, the samples irradiated in the different times present vast differences in the relatively abundance of fatty acids obtained. The highest

decrease was found in C16:1 followed by an increase of C16:0. This modification is directly proportional with the light exposure time.

The relative abundance of the total saturated and unsaturated fatty acids was calculated in the three irradiation times and it is summarized on Figure 16. This was done to better visualize the changes and transformations that have occurred in the fatty acids profile.

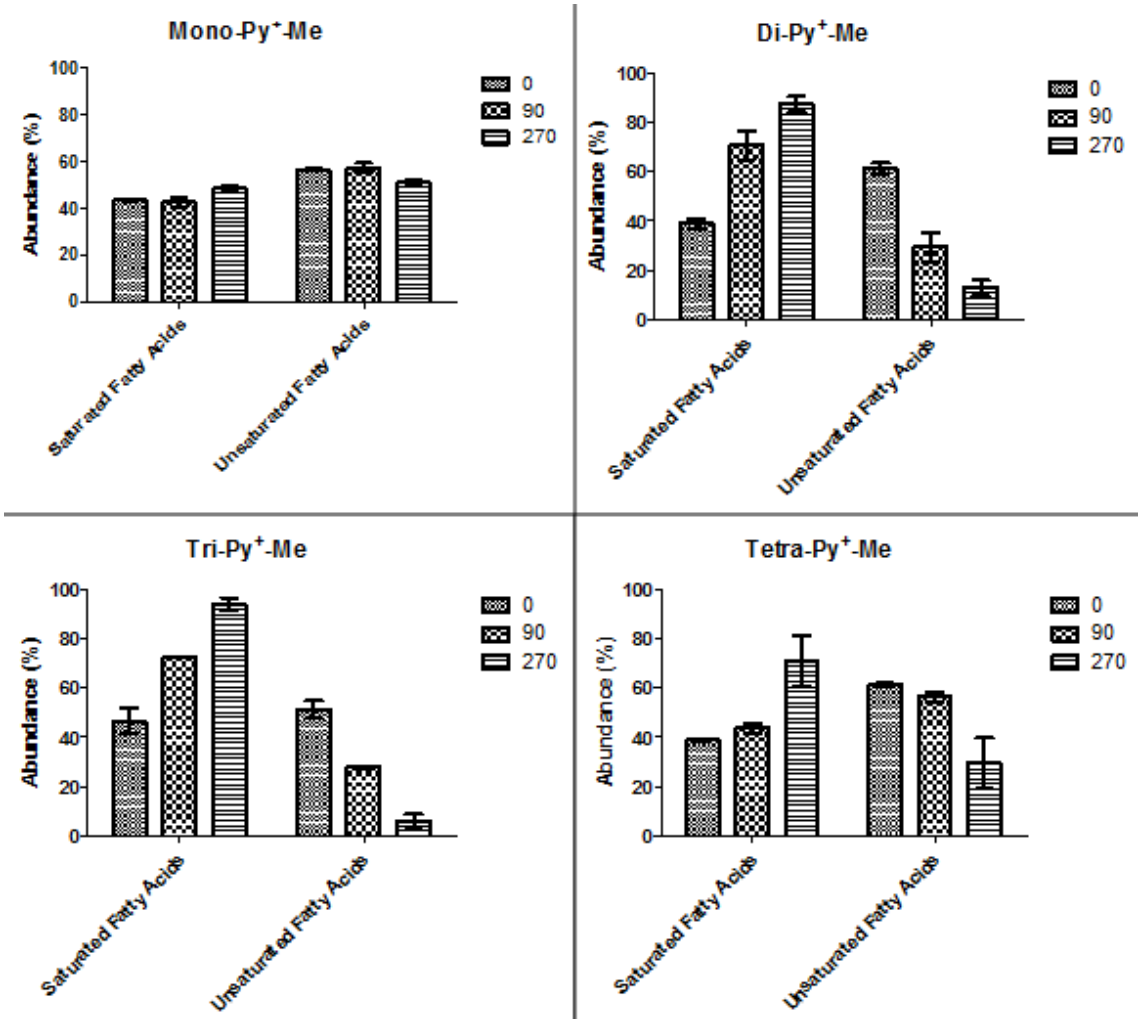

Figure 16 – Relative abudance (%) of saturated fatty acids and unsaturated fatty acids together for each porphyrin in the three irradiation times.

The results using the Mono-Py^{+}-Me porphyrin as PS show non-significant variations of saturated vs unsaturated fatty acids over time. It is possible to undertake, according to this method, that this porphyrin does not have high effectiveness in photodynamic inactivation. However, the three remaining

porphyrins (Di-Py$^+$-Me; Tri-Py$^+$-Me; Tetra-Py$^+$-Me) seem to induce higher variations over the exposure time, consequently more effective in PDI.

The efficiency of the PSs is related with the oxidation of fatty acids and is directly related with the decreased of unsaturated fatty acids. As much is the reduction, more effective is the PS. The changes of unsaturated fatty acids relative abundance, on each irradiation time, are illustrated in Figure 17.

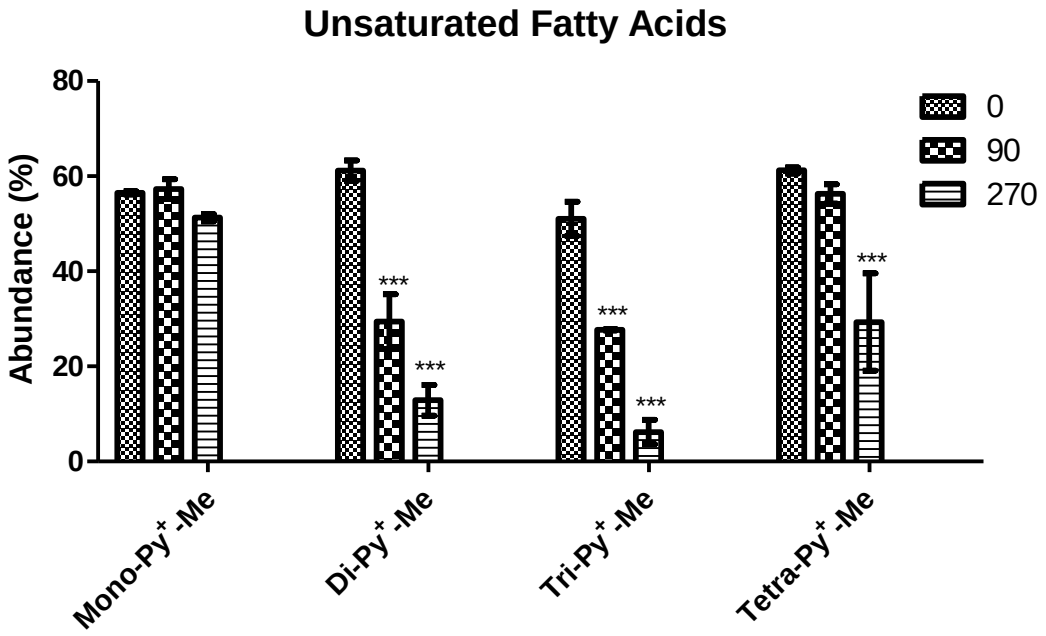

Figure 17 - Relative abudance (%) of unsaturated fatty acid during the three irradiation times. The present values are the averages with ± standard deviation. It was effected aone-way ANOVA with significant difference with Light Control ***, P<0.001 ; * P < 0.05.

The porphyrin Di-Py$^+$-Me reports the higher variances after 90 minutes of light exposure with a decrease of approximately 30 % of unsaturated fatty acids. Tri-Py$^+$-Me also reports high modifications with a decrease of approximately 20 %. Tetra-Py$^+$Me do not show significant changes after 90 min of exposure. After 270 min the results are similar, but the porphyrin Tri-Py$^+$-Me presents the highest decrease in unsaturated fatty acids, approximately 45 %. Di-Py$^+$-Me demonstrated a decrease of approximately 30 %. The porphyrin Tetra-Py$^+$-Me shows a significant decrease of 22 %.

According to these analyses it is possible to establish an order of effectiveness of the porphyrins that is summarized in Table IV.

Table IV - Order of effectiveness established according to fatty acids profile for each porphyrin.

Order	90 minutes	270 minutes
1	Di-Py^{+}-Me	Tri-Py^{+}-Me
2	Tri-Py^{+}-Me	Di-Py^{+}-Me
3	Tetra-Py^{+}-Me	Tetra-Py^{+}-Me
4	Mono-Py^{+}-Me	Mono-Py^{+}-Me

Given these results, one can claim that, among the studied PSs, Mono-Py^{+}-Me is the less effective PS, followed by Tetra-Py^{+}-Me. Di-Py^{+}-Me and Tri-Py^{+}-Me are the most effective PS regarding the analyses of gas chromatography.

4. Cell viability

The previous results give important information about the effect of porphyrins with different charge positions in lipids; however they do not give precise information about the effect of PDI treatment in bacterial viability. In order to accomplish this effect and try to find relations between lipid oxidation and cell survival, viability tests were performed.

The results of the photodynamic inactivation experiments regarding the cell viability of *Aeromonas salmonicida* are summarized in figure 17 and represent the average values with the standard deviation. In all cases the dark and light controls show that the viability of the bacterium (≈ 9 log CFU mL $^{-1}$) was neither affected by light alone nor by the porphyrins used as PSs in the dark at 5.0 µM. In accordance with the previous results on lipid peroxidation, the porphyrin Mono-Py^{+}-Me was not effective against *Aeromonas salmonicida*. The cell viability, during the irradiation time, remained stable along with both dark and light controls (Figure 18).

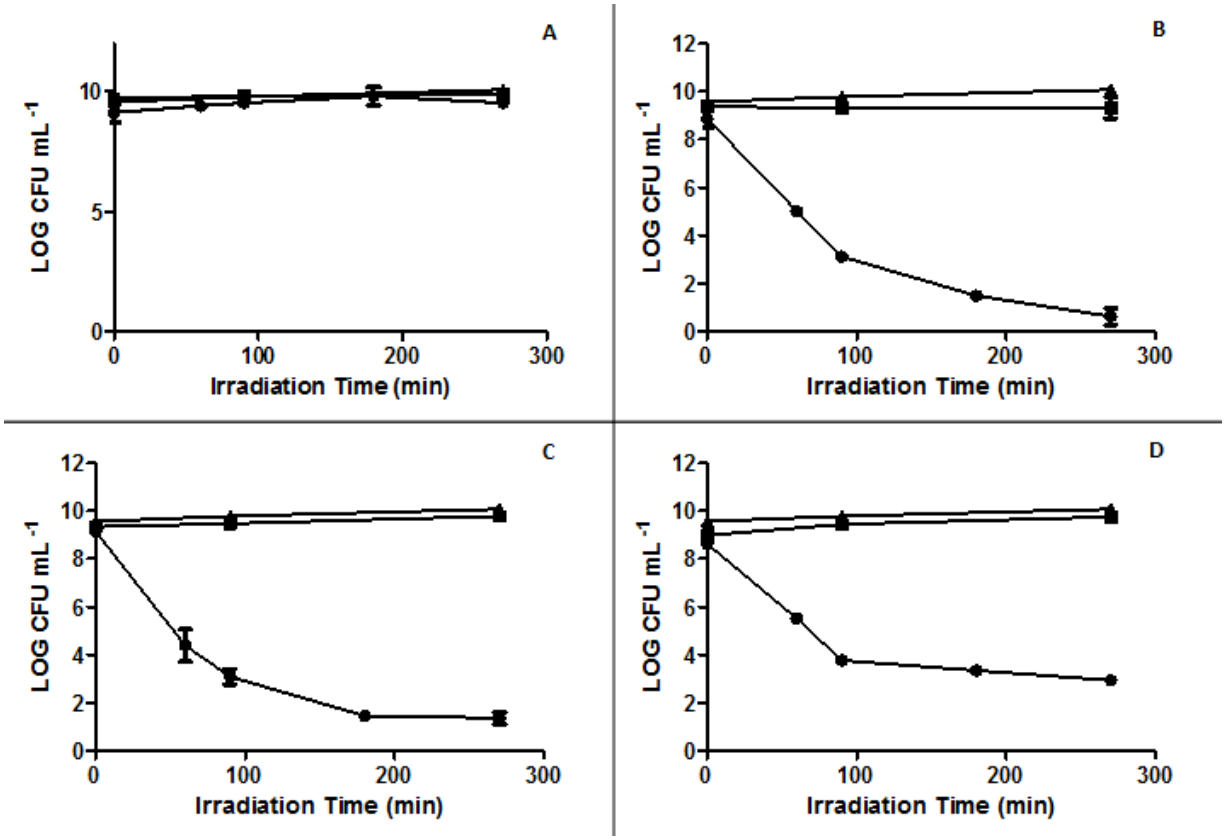

Figure 18 – Viability variation of *A. salmonicida* over the irradiation time for the four porphyrins; A - Mono-Py^{+}-Me; B - Di Py^{+}-Me; C - Tri Py^{+}-Me; D – Tetra- Py^{+}-Me. The samples were irradiated with white light (380–700 nm) with an irradiance of 40 W m−2 for 270 min. Irradiated sample: —●—. Dark control: —■—. Light control: —▲—.

Mono-Py^{+}-Me do not show variances in cell survival during the irradiation time. The cell number remains constant over the period. For the porphyrins Di-Py^{+}-Me and Tri-Py^{+}-Me, the reported results show approximately 6 log reduction on cell survival after 90 min of irradiation with visible light. The experiments using Tetra-Py^{+}-Me show a reduction of 5 logs after 90 min of irradiation. After 270 min the cell viability for Di-Py^{+}-Me was at the lowest levels, with almost complete inactivation. Tri-Py^{+}-Me, after 270 min of irradiation, shows a remarkable reduction of 8 log of colony forming units (CFU). Tetra-Py^{+}-Me shows a big decrease on cell viability at 90 min of light exposure, however, cell viability was only reduced by approximately 1 log from time 90 to time 270 min of irradiation. In three porphyrins that showed photodynamic effect on *Aeromonas salmonicida* the biggest decreased seems to occur after 180 min of light exposure.

Figure 19 compiles the results for all porphyrins in all the different irradiation times. The blue line represents the assays using Mono-Py^+-Me; the orange line represents the assays using Di-Py^+-Me; the green line represents the assays using Tri-Py^+-Me; and the black line represents the assays using Tetra-Py^+-Me. Figure 20 display the total reduction occurred in bacterium viability after 270 min. The changes of cell viability from time 0 to time 270 minutes of irradiation are demonstrated in figure 18. Mono-Py^+-Me do not show variations along the time; Di-Py^+-Me and Tri-Py^+-Me show the highest decreased of bacterium colonies, with a cell survival decreased of approximately 8 log of CFU; Tetra-Py^+-Me reports a reduction of approximately 5 log of CFU.

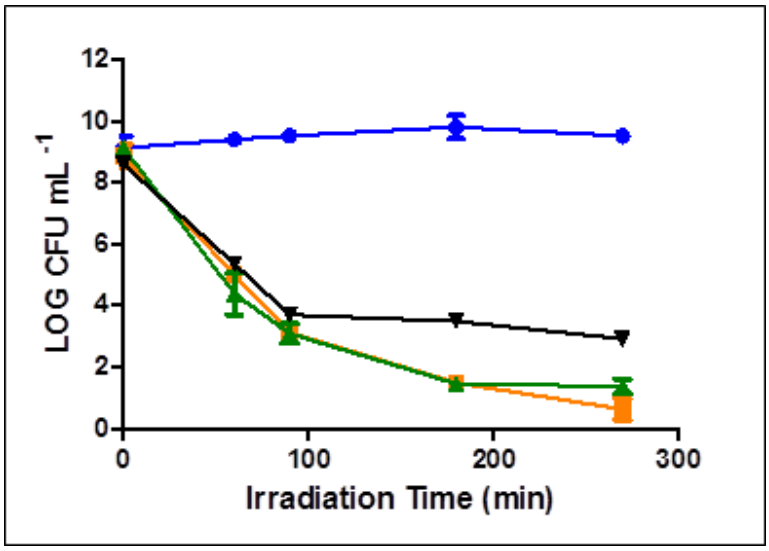

Figure 19 - Viability variation of *A. salmonicida* over the irradiation time for the four porphyrins; . The blue line represents the assays using Mono-Py^+-Me; the orange line represents the assays using Di-Py^+-Me; the green line represents the assays using Tri-Py^+-Me; and the black line represents the assays using Tetra-Py^+-Me

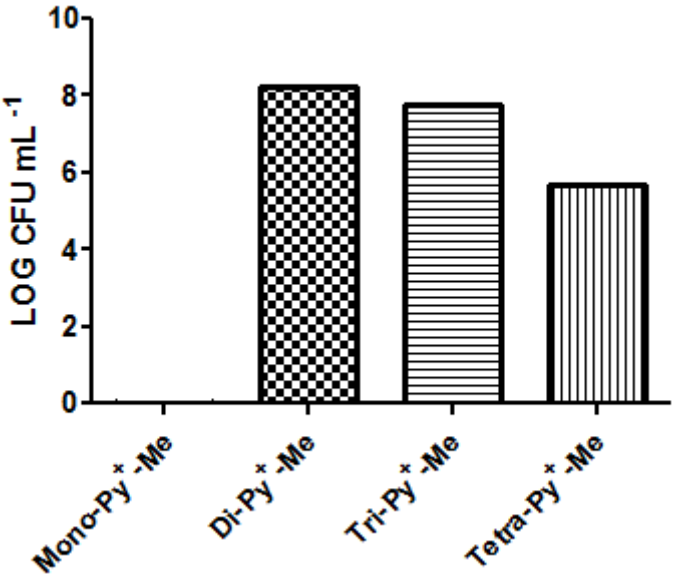

Figure 20 - Total reduction occurred in bacterium viability after 270 min for each porphyrin

According to these results, it is possible to establish an order of effectiveness for the used porphyrins in each time. The establish order is displayed on the Table V.

Table V - Order of effectiveness established according to cellular viability tests.

Order	90 minutes	180 minutes	270 minutes
1	Di-Py$^+$-Me	Di-Py$^+$-Me	Di-Py$^+$-Me
2	Tri-Py$^+$-Me	Tri-Py$^+$-Me	Tri-Py$^+$-Me
3	Tetra-Py$^+$-Me	Tetra-Py$^+$-Me	Tetra-Py$^+$-Me
4	Mono-Py$^+$-Me	Mono-Py$^+$-Me	Mono-Py$^+$-Me

Chapter IV

Discussion

Discussion

Antibiotic resistance is a complex problem that has been in continuous evolution (Barker, 1999). Nowadays, the research for new antimicrobials has face considerable growing costs and pharmaceutic industries have reduced its investment in this field, turning to more profitable drugs. Photodynamic inactivation emerges as a promising alternative to inactivate important microorganisms (Hamblin et al., 2011; Pereira et al., 2014; Maisch et al., 2004; Alves et al., 2009a). Knowing that the photodynamic inactivation of bacteria is, in the current years, an important, efficient and PS-dependent alternative to more conventional approaches (Pereira et al., 2014; Tavares et al., 2010), it was decided to evaluate how different porphyrinic compounds are able to inactivate the bacterium *Aeromonas salmonicida*. The studied bacterium was chosen because of the different composition and organization of bacterial structures, compared to traditional gram-negative bacteria with a complex and well-structured protein s-layer (Thomas and Trust, 1995; Pereira et al., 2014). This work was developed on the assumption that different porphyrins could have different responses on lipids after photo-inactivation of *Aeromonas salmonicida* bacterium, which was observed.

It has been reported by other studies that the photo inactivation efficiency of microorganisms using PSs, in particular porphyrinic types, is dependent on the molecules structure, number of present charges, the nature of the substituted groups in *meso* position and its amphiphilic nature (Pereira et al., 2014; Alves et al., 2009b). A wide range of studies suggests that porphyrins with different charges show different responses against bacteria (Reddi et al., 2002; M. R. Hamblin and Hasan, 2004; Tomé et al., 2004; M. Merchat et al., 1996; Magaraggia et al., 2006; Costa et al., 2008; Arrojado et al., 2011; Pereira et al., 2014). Cationic compounds are more effective to inactive bacteria than neutral derivatives (Alves et al., 2009a; Spesia et al., 2005; Demidova and Hamblin, 2005).

In the present study the same conclusions can be made. Different porphyrins have different responses and the cationic compounds seem to be more effective than neutral derivatives. The control show minor quantities of LOOH, corresponding to a natural baseline that does not vary over time, indicating minor lipid oxidation. Thus results are in accordance with the central principle of Photodynamic Therapy, which say that the photo-oxidation occurs in the

presence of light and a PS together, and never by these factors alone (Costa et al., 2011; Arrojado et al., 2011; Preuß et al., 2014). Accordingly, it can be inferred that the variations that occurred over time, are due to the action of PDI.

The effectiveness of photodynamic therapy is strongly dependent on the used PS (Arrojado et al., 2011). The current study shows the effectiveness of four *meso*-substituted porphyrins with different number of charges in the photo-oxidation of membrane lipids of *Aeromonas salmonicida*. According to the obtained data of formed LOOH, the efficacy of photo-oxidation is not directly related with the number of charges in the porphyrins. Other studies have concluded a directly relation between the number of charges with the increase effectiveness of the therapy. On these studies the porphyrins Tetra-Py^+-Me and Tri-Py^+-Me are reported with the best performance (Caminos et al., 2006; Alves et al., 2009c; Caminos et al., 2006; Debora Lazzeri et al., 2004; Lopes, 2013)

The results of the current study show significant differences regarding the production of LOOH for the different used porphyrins. Other studies, using different porphyrins and other molecules as PSs also showed differences in the efficacy of the therapy (Dai et al., 2010; Hamblin et al., 2011; Alves et al., 2009a; Pereira et al., 2014). On this study it is confirmed that the effectiveness of therapy is strongly dependent on PS choices, but not with a directly relation with the number of charges, porphyrins with 2 and 3 positive charges reported the highest bacterial inactivation.

The porphyrin Di-Py^+-Me after 90 and 270 minutes of irradiation with a visible light demonstrated the highest production of LOOH, followed by the porphyrin Tri-Py^+-Me. The both porphyrins revealed a higher production of lipid hydroperoxides compared to the other porphyrins (p value < 0.001). The porphyrin Tetra-Py^+-Me was expected to lead to the highest production of LOOH because of the highest number of cationic charges (Alves et al., 2009a). However, this compound revealed a low capacity of photo oxidation, regarding the production of LOOH, for both 90 and 270 minutes of irradiation. As expected, Mono-Py^+-Me reported the lowest levels of formed LOOH's (Alves et al., 2009c). A similar study with *Escherichia coli* concluded that Mono-Py^+-Me is the PS that leads to the lowest production of LOOH; Di-Py^+-Me and Tri-Py^+-Me reports the highest production of LOOH; and Tetra-Py^+-Me demonstrates an average production of LOOH (Lopes, 2013)

The FOX II quantifies the LOOH, which are the first formed species in lipid oxidation. However, these products can easily be degraded in the presence of metallic ions, originating secondary oxidation products, such as hydroxyl derivate. The secondary oxidative products cannot be quantified by FOX II. It does not verify if the oxidation products were degraded or upgraded to secondary products, however, it gives valid information about the occurrence or absence of lipid oxidation (Yin and Porter, 2003; Wasylaschuk et al., 2007; Yin et al., 2011).

In short, it was detected oxidation after PDI, leading to a high production of LOOH after 270 minutes of irradiation. This method alone is not adequate to establish and order; giving only an estimate of porphyrins effectiveness.

For a better understanding of membrane lipid oxidation in *Aeromonas salmonicida,* a quantification of fatty acids by Gas Chromatography was performed for each porphyrin in the three irradiation periods. The identified fatty acids are in accordance with previous studies, using a similar approach (Huys et al., 1994).

A decrease of unsaturated fatty acids was observed along the irradiation time. According to literature, the decrease of unsaturated fatty acids represents an evidence of lipid oxidation. (Frankel, 1984; Vick and Zimmerman, 1987). The decrease on monounsaturated fatty acids occur because of modifications caused by it oxidation (Arrojado et al., 2011). Differences in the relative abundance of these fatty acids were expected due to oxidation, but not such large changes. Previously results concluded that the number of positive charges seem to affect the efficiency of Photoinactivation in bacteria. The Tri-Py^{+}-Me-PF porphyrin was the most effective, followed by Di-Py^{+}-Me (Lopes, 2013; Malik et al., 1990; Pereira et al., 2014; Alves et al., 2009a). The current study provides slightly different results, regarding the effectiveness of porphyrins. These dissimilarities may be due to *Aeromonas salmonicida* cell wall (Pereira et al., 2014; Thomas and Trust, 1995; Sára and Sleytr, 2000). A previous study, using the same methodology for *E.coli* show the same variations with lower orders of magnitude (Lopes, 2013). The reason for the observed differences can be the fact that this bacterium has different wall structures, with a complex protein layer as the outer membrane (Sára and Sleytr, 2000). In the present study, the greatest difference occurs from 90 to 270 minutes. One possible explanation is

that the bacterium may have lost or degraded its membrane after 90 minutes. Thus, the lipids would be more vulnerable to oxidation (Rudloff, 1956).

A cellular viability study, using Tetra-Py^+-Me as PS, reported a reduction of 5 log CFU for *Aeromonas salmonicida* According to the study, the highest reduction in CFU occurs within 90 minutes of irradiation. From 90 to 270 minutes there is only a reduction of approximately 1 Log (Pereira et al., 2014).

For these reasons, viability tests were performed for all porphyrins in the three irradiation times. Di-Py^+-Me was the most effective PS with 8 log reduction on cell survival after 180 min of irradiation and complete cell dead after 270 minutes of irradiation. Tri-Py^+-Me also demonstrated 8 log reduction on cell survival after 180 min of irradiation and 10 log reduction on cell survival after 270 min. These results explain the high quantification of unsaturated fatty acids. After 90 min of light exposure the cells had suffered a massive reduction of viability. At this time the lipids are more vulnerable to oxidation (Rudloff, 1956). Tetra-Py^+-Me shows a reduction of 5 logs on cell viability after 90 minutes of light exposure, as has been observed in other study (Pereira et al., 2014). Cell viability using Mono-Py^+-Me remained stable. The viability assays are in accordance with fatty acids quantification and with the lipid hydroperoxides quantification. These results represent evidence that the number of PS charge does not have a direct relation with the oxidation effectiveness, as other study has demonstrated and the effect only happens with 2 or more charges (Lopes, 2013).

Aeromonas salmonicida is a gram-negative bacteria, it presents a proteinaceous layer attached to the outer membrane as the outermost cell wall component. The S-layer is a two-dimensional crystalline tetragonal array of a single protein species, the A protein, which covers the entire bacterial cell, and binds to the O-antigen of the *A. salmonicida* lipopolysaccharide (Pereira et al., 2014). This layer increases the surface hydrophobicity and provides constitutive protection against oxidative damage (Arrojado et al., 2011; Pereira et al., 2014)

The results obtained in this study indicates that the use of Di-Py^+-Me and Tri-Py^+-Me-PF in the combination of visible light is effective to oxidize bacteria, being a viable and environmental friendly alternative to inactivate *A.salmonicida*, as has already been concluded by other studies using different approaches (Lopes, 2013; Magaraggia et al., 2006; Jori and Coppellotti, 2007).

Chapter V

Conclusion

Conclusion

The main goal of the current study was to evaluate the charge effect of four porphyrins, used as PSs, in the photo-oxidation of membrane lipids of the bacterium *Aeromonas salmonicida* and understand the oxidation of these lipids on cellular viability. To evaluate the photo-oxidation two methods were performed, the xylenol orange method (FOX II) and gas chromatography with flame ionization detector (GC-FID). In both methods the results were similar and let to the possibility to establish an order of effectiveness for the four tested porphyrins. The established order was not only the same applying these two methods, but also for the both times of light exposure (90 and 270 min). According to these results the following crescent order on the efficacy of the PS in *Aeromonas salmonicida* is presented: Mono-Py^+-Me < Tetra-Py^+-Me < Tri-Py^+-Me < Di-Py^+-Me.

According to these results, the photo-oxidation is not directly proportional with the number of charges in the PSs, as other studies had been reported. As in another previous study in *E.coli*, the porphyrin mono-cationic is the less efficient and the porphyrins Di-Py^+-Me and Tri-Py^+-Me are the most effective.

A direct relation between the photo-oxidation of membrane lipids with the photo-inactivation in the studied bacterium was observed, as the order of effectiveness established for the porphyrins under the photo-oxidation analysis is the same for the photo-inactivation assays.

This study reinforces that cationic porphyrins are effective to inactivate bacteria and the huge importance of membrane lipids of *Aeromonas salmonicida* as photodynamic therapy targets. However, a proteomic approach seems also important to accomplish, because of the protein composition of the cell wall of this bacterium.

Chapter VI

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